Molecular biological analysis of cell adhesion in *Ashbya gossypii*

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Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. There are no other doctoral examination procedures in progress. The work presented in this thesis is my own work, exceptions were stated. All sources of information have been acknowledged by means of references.

Anke Grünler

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Auswahl und Auswertung des Materials sowie Herstellung des Manuskripts wurde unterstützt von Prof. Dr. J. Wendland und Prof. Dr. J. Wöstemeyer.
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Anke Grünler

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1. Summary

Ashbya gossypii is a filamentous fungus of the family *Saccharomycetaceae*, and about 95% of its genes have a homologue in the yeast *Saccharomyces cerevisiae*. Haploid *S. cerevisiae* can turn into a filamentous growth form upon carbon depletion, and diploids can form pseudohyphae during nitrogen depletion or starvation for amino acids. The expression of adhesion molecules is necessary to maintain the switch from a single cell state to pseudohyphae. Adhesion molecules are cell wall glycoproteins that have a modular structure. In *Candida glabrata* adhesins are encoded by EPA (epithelial adhesion) genes and in *Candida albicans* by ALS (agglutinin-like sequence) genes. *S. cerevisiae* adhesion genes are called *FLO* genes and pheromone induced genes like *FIG2*. The *ScFLO* genes are *FLO1, FLO5, FLO9, FLO10* and *FLO11*. Due to subtelomeric localization of most of them, *ScFLO11* is the only expressed flocculin.

A. gossypii encodes only a limited number of adhesion genes, which are *AgFLO5-1* and *AgFLO5-2*, *AgFLO11* and *AgFIG2*. They are not in synteny with and do not show high similarity to the homologues in *S. cerevisiae*. Genes encoding the transcriptional regulators AgFlo8, AgSfl1 as well as AgTec1 and AgSte12 are present as well. They are localized in synteny to the counterparts in *S. cerevisiae* but do not show high similarity either. The LisH domain of Flo8, the HSF domain of Sfl1, the TEA domain of Tec1 and the STE domain of Ste12 show a high amount of conservation. High similarity of domains indicates conserved functions in DNA binding and complex formation with other proteins, respectively. It could be possible that permanent expression of adhesins in *A. gossypii* is responsible for the filamentous appearance and attachment to the growth substrate. Flocculation gene products in *S. cerevisiae* are of Flo1 type and flocculation and adhesion is repressible with mannose. This is not the case in *A. gossypii* indicating different binding specificities.

Deletion of *A. gossypii* adhesins and most of their transcriptional regulators did not alter the colony phenotype. However, *Agflo8* and *Agsfl1* showed a significant growth delay, and *Agtec1* showed less growth delay on solid media. All three produced small mycelia balls in liquid culture. Microscopic analysis showed a normal but delayed branching pattern, and hyphal phenotypes including aerial hyphal aggregations did not differ from the precursor strain. Attachment was not impaired either. The mentioned mutants

sporulated in an abundant manner compared to the precursor strain but spore phenotypes were normal. Growth delay of a *FLO8* and *SFL1* deletion occurred as well when deleted in another *Eremothecium* strain, suggesting the deletion as the cause for it. Reporter gene assays using *StlacZ* controlled by promoters of *AgFLO5-1, AgFLO5-2, AgFLO11, AgFIG2* and their transcriptional regulators *AgFLO8* and *AgSFL1* were carried out, and expression in the strains *Agleu2, Agflo8, Agsfl1, Agfig2, Agtec1* and *Agste12* was measured quantitatively using â-galactosidase assays. Expression of *AgFLO5-1, AgFLO11* and *AgSFL1* was below the defined threshold of expression of 10 MU in *A. gossypii* In the *AgSFL1* deletion strain, a derepression of *AgFLO5-2, AgFLO8* and *AgFIG2* was observable and deletion of *AgFLO8* resulted in similar derepression. Studying the promoter of *AgSFL1*, several Flo8 binding sites could be identified. It is possible that AgFlo8 regulates *AgSFL1* gene expression, and AgSfl1 represses expression of *AgFLO5-2, AgFLO8* and *AgFIG2*. The involvement of AgFlo8 in expression of *AgSFL1* explains similar phenotypes. The *AgFLO8* and *AgSFL1* deletion mutants seemed to possess certain insensitivity to elevated sugar levels, indicating non sufficient stress response in the absence of AgSfl1.

An explanation of the growth delay of both strains could be that, in addition to the upregulated expression of *AgFLO5-2, AgFLO8* (for *Agsfl1*) and *AgFIG2,* other Sfl1 controlled genes might be derepressed as well and lead to the observed phenotype. A hypothesis could be the incorporation of the named adhesins into the cell wall or the higher expression of certain enzymes or components, which lead to a denser cell wall and therefore reduced endocytosis or growth speed.

Overexpression of *AgSFL1* resulted in a wild-type-like phenotype. A possible explanation for that could be the constant suppression of the controlled genes under normal growth conditions.

Similar to *C. albicans*, a C-terminal GFP fusion to AgSfl1 shows that the protein is localized in nuclei. This correlates with the presence of a nuclear localization domain.

In contrast to *S. cerevisiae* where pheromone stimulation is necessary, *AgFIG2* seems to be permanently expressed at high levels. Reporter gene assays showed that AgTec1 is the major transcriptional activator of *AgFIG2,* since its expression is absent in an *AgTEC1* deletion strain. In *S. cerevisiae* Ste12 is involved in *FIG2* expression which is not the case in *A. gossypii*, since expression levels are not altered in an *AgSTE12* deletion mutant. This data suggests different regulation between the two organisms.

Examination of conservation of transcription factor binding sites between *A. gossypii*

and *S. cerevisiae* were carried out using reporter gene assays. AgCts2, a chitinase specifically expressed in sporulation zones, possesses 2 binding sites for Tec1 in its gene promoter, based on comparison with the *S. cerevisiae* protein properties. The expression of *StlacZ* under control of the *AgCTS2* promoter is absent in an *AgTEC1* deletion strain, confirming the conservation of the Tec1 binding sites between the two organisms. Out of 4726 protein coding genes in the genome of *A. gossypii* 1737 (36.8%) show one or more Tec1 binding sites in their promoter when it was searched after CATTC(C/NN) but only 16.6% when using the consensus sequence CATTC(C/YY) like for ScTec1. The majority of all Tec1 regulated genes carries the site CATTCC in their promoter and possesses only a single binding site. This indicates conservation of this consensus between *S. cerevisiae* and *A. gossypii*, but conservation of binding sites needs to be examined further. The involvement of Tec1 in transcriptional regulation of that many genes in *A. gossypii* explains the phenotype of reduced growth in a *TEC1* deletion mutant, where there might be several Tec1 controlled genes involved in normal growth.

AgRib3, an enzyme in the riboflavin production pathway, possesses three potential Ste12 binding sites in its promoter. *StlacZ* expression driven by the *AgRIB3* promoter was very low in an *AgSTE12* deletion strain, indicating conservation of Ste12 binding sites between *A. gossypii* and *S. cerevisiae*.

A. gossypii encodes also other cell wall glycoproteins that could be responsible for surface attachment. In *S. cerevisiae* there are cell wall mannoproteins encoded by *CWP1* YKL096W and *CWP2* YKL096W-A, which bear a GPI anchor and are necessary for cell wall integrity. *A. gossypii* bears no homologue to *ScCWP2* but 7 copies of *CWP1* paralogs in its genome. Three of the *AgCWP1* genes seem not to have any involvement of Tec1 or Ste12 transcription factors in their expression. These proteins could independently or together with the flocculation genes mediate attachment to surfaces in *A. gossypii* They could also contribute to adhesion in *Agtec1* or *Agste12* deletion mutants and need to be further examined.

1. Zusammenfassung

Ashbya gossypii ist ein filamentöser Pilz der Familie *Saccharomycetaceae*, und für etwa 95% seiner Gene ist ein homologes Gen in der Hefe *Saccharomyces cerevisiae* zu finden. Im haploiden Stadium kann *S. cerevisiae* bedingt durch Kohlenhydratmangel eine filamentöse Wachstumsform ausbilden, und im diploiden Stadium können Pseudohyphen während Stickstoffmangels oder des Fehlens einzelner Aminosäuren gebildet werden. Für den Wechsel vom Einzellstadium zum pseudohyphalen Wachstum ist die Expression von Adhäsionsmolekülen notwendig. Adhäsionsmoleküle sind Zellwandglykoproteine und zeigen modulare Struktur. Adhäsine in *Candida glabrata* werden von EPA (epithelial adhesion)- Genen und in *Candida albicans* von ALS (agglutinin-like sequence)- Genen kodiert. Adhäsin-Gene in *S. cerevisiae* tragen den Namen *FLO*-Gene bzw. pheromon-induzierte Gene wie zB. *FIG2*. Die *ScFLO*-Gene bestehen aus *FLO1, FLO5, FLO9, FLO10* und *FLO11*. Die Mehrzahl weist subtelomere Lokalisierung auf, womit *ScFLO11* das einzige exprimierte Flockulin darstellt.

A. gossypii besitzt lediglich eine begrenzte Anzahl Adhäsionsmoleküle, welche von *AgFLO5-1* und *AgFLO5-2*, *AgFLO11* und *AgFIG2* kodiert werden. Sie befinden sich nicht in Syntänie zu den homologen Genen von *S. cerevisiae* und weisen keine hohe Ähnlichkeit zu diesen auf. Sowohl die Transkriptionsregulatoren AgFlo8, AgSfl1 als auch AgTec1 und AgSte12 sind vorhanden. Sie weisen ebenfalls geringe Ähnlichkeit zu den Homologen in *S. cerevisiae* auf, aber ihre Gene befinden sich in Syntänie zu diesen. Die LisH Domäne von Flo8, die HSF Domäne von Sfl1, die TEA Domäne von Tec1 und die STE Domäne von Ste12 weisen hingegen einen hohen Grad der Konservierung auf. Hohe Ähnlichkeiten von Domänen deuten koservierte Funktionen an wie zB. DNA-Bindung und Komplexbildung mit anderen Proteinen. Es wäre möglich, dass dauerhafte Expression der Adhäsine in *A. gossypii* zu filamentösem Wachstum und zum Anhaften an das Wachstumssubstrat führen. Die Flockulierungsmoleküle von *S. cerevisiae* sind vom Flo1- Typ, und Flockulierung und Adhäsion können mit Mannose unterdrückt werden. Dies ist nicht der Fall in *A. gossypii*, was auf unterschiedliche Bindungsspezifitäten hinweist.

Deletion sowohl der Adhäsine als auch deren Transkriptionsregulatoren in *A. gossypii* führte nicht zu einer Änderung des Koloniephänotyps mit Ausnahme von *Agflo8*,

Agsfl1 und *Agtec1* Deletion von *AgFLO8* und *AgSFL1* führte zu signifikanter und Deletion von *AgTEC1* zu weniger starker Wachstumsverzögerung auf Festmedium. Alle drei Deletionsmutanten bildeten kleine Hyphenbällchen in Flüssigkultur aus. Die mikroskopische Analyse zeigte normales wenn auch verzögertes Verzweigungsmuster des Myzeliums mit unverändertem Hyphenphänotyp. Lufthyphenaggregationen wiesen keinen Unterschied zum Wildtyp auf, und das Anhaftungsverhalten blieb ebenfalls unverändert. Die genannten Deletionsmutanten zeigten vermehrte Sporenbildung verglichen mit dem Wildtyp, jedoch waren Sporenphänotypen unverändert. Wachstumsverzögerung bedingt durch Deletion von *FLO8* und *SFL1* trat auch in einem *Eremothecium* Stammhintergrund auf, was die Abwesenheit der Gene als Ursache unterstreicht. Experimente mit *StlacZ* als Reportergen unter Kontrolle der Promotoren von *AgFLO5-1, AgFLO5-2, AgFLO11, AgFIG2* und deren transkriptioneller Regulatoren *AgFLO8* und *AgSFL1* wurden durchgeführt. *LacZ* Expression wurde nach Transformation der Stämme *Agleu2, Agflo8, Agsfl1, Agfig2, Agtec1* und *Agste12* quantitativ mit einem â-Galaktosidase Ansatz bestimmt. Expression von *AgFLO5-1, AgFLO11* und *AgSFL1* befand sich unterhalb des definierten Expressionsgrenzwertes von 10 MU in *A. gossypii* Deletion von *AgSFL1* bewirkte eine Derepression von *AgFLO5-2, AgFLO8* und *AgFIG2* und Deletion von *AgFLO8* verhielt sich ähnlich. Im Promotor von *AgSFL1* konnten mehrere Flo8 Bindestellen gefunden werden. Dies lässt den Schluss zu, dass AgFlo8 die Genexpression von *AgSFL1* reguliert und AgSfl1 die Expression von *AgFLO5-2, AgFLO8* und *AgFIG2* reprimiert. Die Beteiligung von AgFlo8 an der Expression von *AgSFL1* erklärt ähnliche Phänotypen. Die Deletionsmutanten *AgFLO8* und *AgSFL1* schienen eine gewisse Insensitivität gegenüber erhöhten Zuckermengen aufzuweisen, was auf unzureichende Stressabwehr in Abwesenheit von AgSfl1 hinweist.

Die Ursache der Wachstumsverzögerung beider Stämme kann darin liegen, dass neben erhöhter Expression von *AgFLO5-2, AgFLO8* (in *Agsfl1*) und *AgFIG2* möglicherweise andere Sfl1 kontrollierte Gene ebenfalls dereprimiert werden und zum beschriebenen Phänotyp führen. Eine Hypothese könnte der Einbau der genannten Adhäsine in die Zellwand darstellen oder die erhöhte Expression von Enzymen oder Komponenten die zu einer dichteren Zellwand und somit reduzierten Endozytose oder Wachstumsgeschwindigkeit führen.

Überexpression von *AgSFL1* resultierte in wildtypartigem Phänotyp. Eine mögliche Erklärung dafür stellt die konstante Suppression der von Sfl1 kontrollierten Gene unter normalen Wachstumsbedingungen dar.

Wie bereits für *C. albicans* beschrieben, zeigte eine C-terminale GFP Markierung von AgSfl1 die Lokalisierung des Proteins im Zellkern, korrelierend mit dem Auftreten eines Kernlokalisierungs-Signals.

Im Gegensatz zur notwendigen Pheromon-Stimulierung in *S. cerevisiae* scheint *AgFIG2* permanent in hohem Maße exprimiert zu sein. Reportergen-Experimente zeigten, dass AgTec1 der wichtigste Transkriptionsaktivator von *AgFIG2* ist, da keine *FIG2* Expression bei *AgTEC1* Deletion nachweisbar war. Ste12 ist beteiligt an der *FIG2* Expression in *S. cerevisiae*. Dies ist nicht der Fall in *A. gossypii*, da dessen Expressionsniveau bei *AgSTE12* Deletion unverändert blieb. Diese Ergebnisse deuten auf unterschiedliche Regulation in beiden Organismen hin. Unter Anwendung von Reportergen-Konstrukten wurde die Konservierung von Transkriptionsfatorbindestellen zwischen *A. gossypii* und *S. cerevisiae* untersucht. AgCts2, eine Chitinase mit spezifischer Expression in sporulierendem Myzel, weist zwei Tec1 Bindestellen im Genpromotor auf. Dies basiert auf Vergleichen mit Erkenntnissen der *S. cerevisiae* Proteinbindung. Bei *AgTEC1* Deletion fehlte die Expression von *StlacZ* unter Kontrolle des *AgCTS2* Promotors. Dies deutet auf konservierte Tec1 Bindestellen in beiden Organismen hin. Das Genom von *A. gossypii* enthält 4726 Gene, die für Protein kodieren. Von diesen weisen 1737 (36.8%) eine oder mehrere Tec1 Bindestellen im Promotor auf, wenn CATTC(C/NN) als Konsensus angenommen wurde. Nur 16.6% zeigen Tec1 Regulierung wenn die Konsensussequenz CATTC(C/YY), welche für ScTec1 bestimmt wurde, angewendet wurde. Die Mehrheit aller Tec1 regulierter Gene besitzen die Bindesequenz CATTCC und zeigen nur eine einzelne Bindestelle im Promotor. Dies deutet auf konservierte Tec1 Bindestellen zwischen *S. cerevisiae* und *A. gossypii* hin, jedoch muss die Konservierung der Konsensus Sequenzen weiterhin untersucht werden. Die Beteiligung von Tec1 an transkriptioneller Regulation vieler Gene in *A. gossypii* erklärt den Wachstumsphänotyp in der Deletionsmutante, wobei verschiedene Tec1 regulierte Gene an normalem Wachstum beteiligt sein können.

AgRib3, ein Enzym beteiligt an der Riboflavinbiosynthese, besitzt drei Ste12 Bindestellen im Genpromotor. Die Expression von *StlacZ* unter Kontrolle des *AgRIB3* Promotors war sehr niedrig in der *AgSTE12* Deletionsmutante. Dies deutet auf Konservierung der Ste12 Bindestellen zwischen *A. gossypii* und *S. cerevisiae* hin.

Im Genom von *A. gossypii* sind noch andere Zellwandglykoprotein kodierende Gene zu finden, welche für Oberflächenadhäsion verantwortlich sein könnten. In *S. cerevisiae* sind Zellwand-Mannoproteine vorhanden, die von *CWP1* (YKL096W) und *CWP2* (YKL096W-A) kodiert werden und einen GPI Anker aufweisen. Sie sind notwendig für die Integrität der Zellwand. *A. gossypii* kodiert kein Homologes zu *ScCWP2* jedoch 7 Paraloge des *CWP1* Gens. Drei der *AgCWP1* Gene scheinen keine Beteiligung von Tec1 oder Ste12 an deren Expression aufzuweisen. Diese Proteine könnten unabhängig von oder zusammen mit den Flockulierungsproteinen die Anheftung an Oberflächen in *A. gossypii* bewirken. Sie könnten auch zur Adhäsion in *Agtec1* oder *Agste12* Deletionsmutanten beitragen, was jedoch weiter untersucht werden muss.

2. Introduction

2.1. The fungus *Ashbya gossypii*

Ashbya gossypii, a plant pathogen on cotton and citrus fruits, belongs to the family *Saccharomycetaceae* and about 95% of its genes have a homologue in *Saccharomyces cerevisiae* (Ashby and Nowell 1926, Dietrich *et al.* 2004, Prillinger *et al.* 1997). Despite the high genetic similarity the phenotypic difference between these two fungal species is very high. *Saccharomyces cerevisiae* occurs in a single cell state under normal conditions whereas the phenotype of *A. gossypii* is mostly filamentous. The haploid fungus produces mononuclear needle shaped spores. Upon germination isotropic growth occurs, that leads to a spherical germ cell. Following the emergence of a first germ tube, a second one occurs at the opposite site of the germ cell, initiating the polarized growth phase, which is characteristic for the young mycelium. The germ tubes are separated from the germ cell by septa. The juvenile stadium includes lateral branching and lasts up to 24 hours. After this time the mycelium enters a mature state that is characterised by dichotomous tip branching. The oldest hyphae of the mycelium initiate spore production. A scheme of the life cycle of *A. gossypii* is shown in Figure 1 (based on Wendland, J. and Walther, A. (2005)).

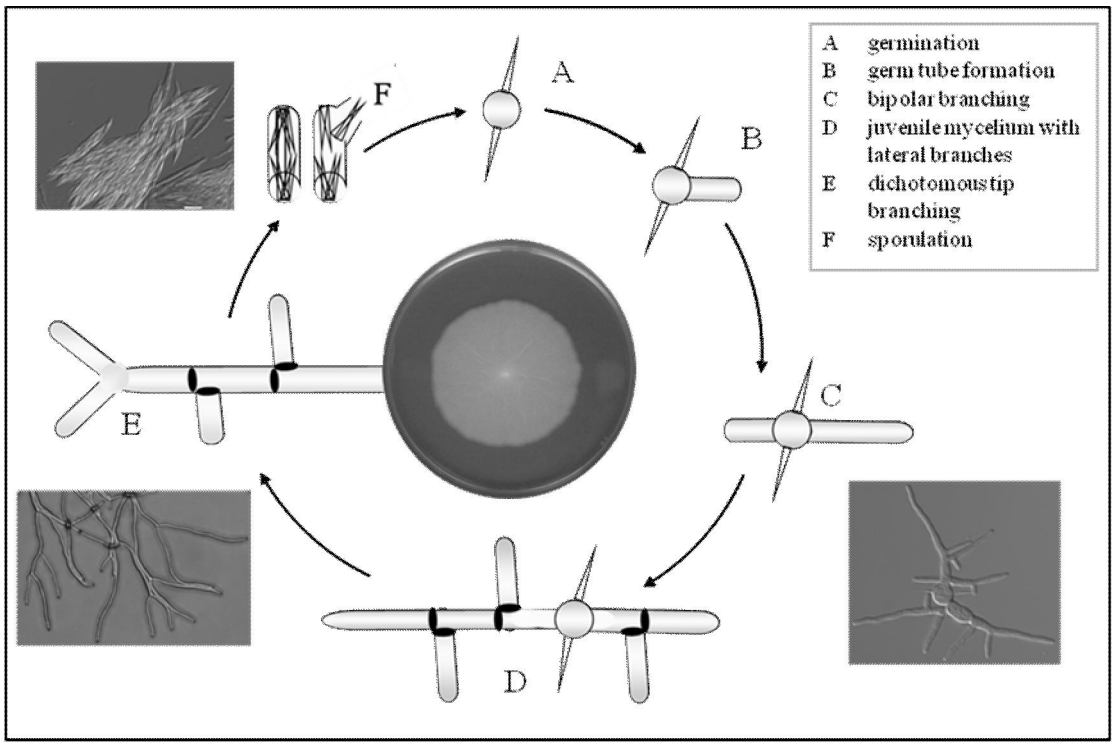


Figure 1. Life cycle of *Ashbya gossypii*. A picture of a colony on full medium after 7 days of growth at 30°C is shown in the middle. The stages are indicated with letters and pictures of juvenile mycelium with lateral branches, mature mycelium with Y-shaped tip branching and spores are shown. Descriptions of single stages are in the text.

Hyphae fragment at septal sites and form endospore containing sporangia in which the spores are bound together by filaments. The hyphae around the endospores stick together and form synnemata like structures that are exposed into the air (reviewed in Wendland, J. and Walther, A. 2005). The mature mycelium produces riboflavin during sporulation that is commercially used (Guilliermond *et al.* 1935, Stahmann *et al.* 2000).
A. gossypii has seven chromosomes with 4726 protein encoding genes. With a total genome size of 9.2 megabases the average protein-coding gene size is 1.9 kb leaving an average distance between open reading frames of 341 bp. *A. gossypii* possesses only 221 introns in its compact genome with a GC content of about 52% (Dietrich, F.S. *et al.* 2004; Gattiker, A. *et al.* 2007). In comparison to *S. cerevisiae* there are 333 genes in *A. gossypii* that are translated with higher efficiency and are enriched in genes involved in cell cycle processes. There are 552 *A. gossypii* genes that possess a lower translational efficiency compared to *S. cerevisiae*. Those are enriched in genes involved in metabolic processes. *A. gossypii* as a multicellular organism with a more complicated cell cycle might have need for higher translational efficiency of cell cycle genes. Unicellular *S. cerevisiae* on the other hand shows a faster growth that correlates with the need for gene products for metabolic processes (Jiang, H. *et al.* 2008).
A. gossypii can be transformed by homologous recombination, and plasmids bearing the autonomously replicating sequence (ARS) elements of *S. cerevisiae* can be expressed (Wright, M.C. and Philippsen, P. 1991; Steiner, S. *et al.* 1995, Wendland, J. *et al.* 2000).

2.2. Molecular basics of adhesion

During the life cycle of *Ashbya gossypii* there are only two single cell states, one during spore germination and the other one after formation of sporangia via hyphal fragmentation. *S. cerevisiae* on the other hand occurs under normal condition in a single cell state. During nitrogen depletion or starvation for amino acids diploids can form pseudohyphae, which represent a filamentous growth form (Gimeno *et al.* 1992; Braus *et al.* 2003). Haploids can grow filamentously upon carbon depletion (Roberts, R.L. and Fink, G.R. 1994). Unipolar instead of axial budding in haploid cells or bipolar budding in diploid cells and the preservation of attachment between mother and daughter cell as well as their elongated shape characterise this growth form (Gimeno *et al.* 1992; Roberts, R.L. and Fink, G.R. 1994). The expression of adhesion molecules is necessary to maintain the switch from a single cell state to pseudohyphae (Miki, B.L.A. *et al.*

1982; Lo, W.S. and Dranginis, A.M 1998). These cell wall glycoproteins are present on the surface of the cell of many fungi and mediate the attachment of cells with each other or with abiotic and biotic surfaces (Miki *et al.* 1982; Lo, W.S. and Dranginis, A.M 1998; Cormack *et al.* 1999). In *Candida albicans* adhesins are encoded by ALS (agglutinin-like sequence) genes and in *Candida glabrata* there are EPA (epithelial adhesion) genes present (Hoyer, L.L. *et al.* 1995; Cormack *et al.* 1999). In these pathogens the molecules are important for interaction with host tissue. Adhesins share a common structure. The N-terminal part maintains binding to certain peptides or sugar residues (Hoyer, L.L. *et al.* 1998; Kobayashi, O. *et al.* 1998). It is followed by a central domain consisting of serine- and threonine-rich peptide repeats that undergo posttranslational glycosylation. The C-terminal domain bears a GPI anchor for securement of the protein on the cell surface by covalent binding to â-1, 6-glucans of the cell wall (Bony, M. *et al.* 1997). The N-terminal secretory sequence is cleaved off while transferring the protein through the secretory pathway (Hoyer, L.L. *et al.* 1998). The binding to cell surface peptides or sugar residues is supposed to be lectin-like whereas the binding to abiotic surfaces seems to occur via hydrophilic and hydrophobic interactions mediated by the glycosylated central domain (Miki, B.L.A. *et al.* 1982; Kobayashi, O. *et al.* 1998, Guo, B. *et al.* 2000). *S. cerevisiae* has a group of adhesion genes called *FLO* genes consisting of *FLO1, FLO5, FLO9, FLO10* and *FLO11*. Expression of *FLO1* leads to strong cell-cell adhesion (flocculation) whereas Flo5, Flo9 and Flo10 result only in weak clumping. *FLO10* and *FLO11* expression is necessary for adherence to abiotic surfaces rather than cell-cell attachments (Guo, B. *et al* 2000; Govender, P. *et al.* 2008). Amino acid sequences of Flo1 and Flo5 are 93% identical, Flo1 and Flo9 share a 94% identity and Flo1 is 44% identical to Flo10. Flo11 on the other hand shares only 22% identity with Flo1 (Lo, W.S. and Dranginis, A.M. 1996; MegAlign ClustalW). Adhesion under starvation conditions allows penetration of substrates and foraging for nutrition while flocculation keeps cells protected from environmental stress and allows floating away from stress environments. Important activators of these genes in *S. cerevisiae* are among others Flo8 and a complex of Tec1 and Ste12 (Kobayashi, O. *et al.* 1996; Liu, H *et al.* 1993). A Flo8 ortholog has been described as an inducer of hyphal-specific gene expression in *C. albicans*, too (Cao, F. *et al.* 2006). A negative regulator of flocculation is Sfl1 in complex with the corepressors Tup1 and Ssn6 that binds to the conserved motif AGAAAxxT-n-GTTCTT with variable length of n (Fujita, A. *et al.* 1989; Conlan, R.S. and Tzamarias, D. 2001).

All flocculation genes of *S. cerevisiae* except *FLO11* are situated in subtelomeric regions, leaving it the only expressed flocculin whereas the others remain silent. But even during nitrogen depletion the expression of *FLO11* is metastable due to stochastic changes between expression and silencing, depending on the action of chromatin remodelling proteins Hda1. Other flocculins can be expressed in case of the occurrence of loss of function mutations in the IRA1 or IRA2 genes (Gottschling, D.E. *et al.* 1990; Guo, B. *et al.* 2000; Halme, A. *et al.* 2004). The subtelomeric localisation is also a reason for increased recombination frequencies between flocculation genes of the same or of different chromosomes. The internal repeats that trigger crossing over and homologous recombination are highly conserved among flocculation genes. The conservation applies to the DNA sequence but also to the amino-acid similarity that is not conserved at DNA level. Recombination events might lead to new adhesion properties in changing environments or contribute to evasion of the host immune response in case of pathogenic fungi (Verstrepen, K.J. *et al.* 2004, Verstrepen, K.J. *et al.* 2005). In *C. glabrata* the only expressed agglutinin is *EPA1*. *EPA2* till *EPA5* are localised in subtelomeric regions and therefore silent like many surface glycoprotein encoding genes in *Trypanosoma, Pneumocystis* and *Plasmodium* (De la Penas, A. *et al.* 2003).

S. cerevisiae adhesion can be divided in subcategories called Flo1-type and NewFlo-type. Proteins of the Flo1-type bind only mannose residues whereas NewFlo-type molecules bind mannose, glucose and maltose (Stratford, M. and Assinder, S. 1991, Kobayashi, O. *et al* 1998). The N-terminal binding domain of *C. glabrata* epithelial adhesins contains a pentapeptide that is conserved among *S. cerevisiae* flocculins (Zupancic, M.L. *et al.* 2008). A tryptophan residue is involved in mannose recognition by all Flo proteins, and Flo11-dependent flocculation is inhibited by a concentration of 70 mM mannose. NewFlo-type flocculation requires a leucin at the same position for binding to mannose and glucose (Kobayashi, O. *et al.* 1998, Bayly, J.C. *et al.* 2005). It has also been described that Flo11 has ability for homotypic binding like it is known for Als proteins in *C. albicans* (Douglas *et al.* 2007).

Another class of adhesion molecules in *S. cerevisiae* contains the protein Fig2, which is expressed upon pheromone stimulation in mating conditions. It is necessary for cell fusion, agglutination and cell integrity during mating (Zhang, M. *et al.* 2002). The protein possesses an N-terminal secretion signal and a C-terminal GPI anchor addition signal and is highly O- and N-glycosylated (Erdman, S.E. *et al.* 1998, Huang, G. *et al.*

2009). A MAPK cascade with G-protein coupled receptors Ste2 or Ste3 is involved in its expression.

A. gossypii encodes only a limited number of adhesion genes, which are not localized close to telomeres. There are two genes encoding Flo5, *AgFLO5-1* and *AgFLO5-2*, which are transcribed in inverted orientation. Further *AgFLO11* and *AgFIG2* can be found, but no homologue to *FLO1, FLO9* or *FLO10* is present. Adhesin genes are not in synteny with the homologues in *S. cerevisiae*. Genes encoding the transcriptional regulators *AgFLO8, AgSFL1* as well as *AgTEC1* and *AgSTE12* are present as well and localized in synteny to the counterparts in *S. cerevisiae*. *A. gossypii* adhesins and their transcriptional regulators do not possess a high similarity to the homologues in *S. cerevisiae*. Since expression of adhesins enable *S. cerevisiae* to attach to surfaces and develop a filamentous growth form, it could be possible that permanent expression of adhesins in *A. gossypii* is responsible for the filamentous appearance and attachment to the growth substrate. *A. gossypii* encodes also other cell wall glycoproteins that could be responsible for surface attachment. In *S. cerevisiae* there are cell wall mannoproteins encoded by *CWP1* YKL096W and *CWP2* YKL096W-A which bear a GPI anchor and are necessary for cell wall integrity, since a deletion of one or both lead to cell wall structure and permeability defects (Van der Vaart, J.M. *et al.* 1995). *A. gossypii* bears no homologue to *ScCWP2* but 7 copies of *CWP1* paralogs in its genome.
Yeasts can even adhere to surfaces without functional *FLO11* with the help of glucan and chitin in their cell wall. This occurs in a Swi5/Egt2/Cts2 dependent manner, where *EGT2* encodes an endoglucanase and *CTS2* an endochitinase (Pan, X. and Heitman, J. 2000).

2.3. Regulation of adhesin expression

FLO11 expression occurs in diploid yeast cells as a response to nitrogen starvation and leads to pseudohyphal development and agar invasion in foraging for nutrients. Haploid yeast cells become able to grow invasive upon *FLO11* expression during glucose- or amino acid starvation (Lo, W.S and Dranginis, A.M 1998). Haploid yeast cells can be induced to grow filamentously by isoamyl alcohol, a product of yeast metabolism, diploid pseudohyphal growth is enhanced in presence of ethanol (Lorenz, M.C. *et al.* 2000 a).

Many signalling cascades are implicated in regulating *FLO11* expression in *S. cerevisiae* and not much is known for *A. gossypii* For this reason the following description of gene regulation focuses mostly on the situation in *S. cerevisiae* Signalling cascades include the Ras-cAMP pathway (Lorenz, M.C and Heitman, J. 1997) and the MAPK-dependent filamentous growth pathway (Liu, H *et al.* 1993), but also the main glucose repression pathway (Vyas, V.K. *et al.* 2002) and the Target of Rapamycin (TOR) pathway are considered important (Cutler, N.S. *et al.* 2001). Several pathways can converge in regulation of *FLO11* expression since its promoter is 3.5 kb long with 4 upstream activating and 9 upstream repressing sequences. Flo8 has a dual binding site within a 200 bp fragment in the *FLO11* promoter whereas Ste12 acts on 4 upstream activating sequences (UAS) (Rupp, S. *et al.* 1999). The PKA pathway seems responsible for unipolar budding but not for cell elongation, which is in responsibility of the MAPK pathway component Ste12 (Pan, X. and Heitman, J. 1999).

2.3.1. The MAPK pathway dependent adhesin expression

Msb2 as a stress sensor, the ammonium permease Mep2, that becomes activated upon nitrogen starvation, and the osmosensor Sho1 seem to be the upstream receptors in the MAPK-pathway in *S. cerevisiae*The signalling mucin Msb2 recruits Sho1 and Cdc42 via direct interaction to the filamentous growth pathway (Gagiano, M. *et al.* 1999; Cullen, P.J. *et al.* 2004). In the MAPK pathway Ras2 becomes activated after signal reception and turns Cdc24 in an active state. This molecule activates Cdc42, which, together with Bmh1/2, in turn interacts with the protein kinase Ste20 by replacing the negative regulator Hsl7. Further the MAPK cascade, consisting of Ste11, Ste7 and Kss1, gets activated by Ste20. Unphosphorylated Kss1 blocks the activity of the transcriptional activator Ste12 by interacting with it and the two factors Dig1 and Dig2. Phosphorylated Kss1 attaches a phosphate residue to Ste12 and Dig1/2, which causes their release and the release of the Ste12 repression. Kss1 phosphorylates Cdc25 as well, which in turn activates Ras2 leading to a positive feedback loop and enhancement of the signal (reviewed in Gancedo, J.M. 2001; Sengupta, N. *et al.* 2007). The *STA1* gene promoter has a high homology to the promoter of *FLO11* and a common regulation can be anticipated. For *Saccharomyces diastaticus* it is postulated, that in absence of glucose, Ste12 and Tec1 bind to the FRE in UAS2-1 and recruit the Swi/Snf complex to the promoter of *STA1* This complex promotes binding of Flo8 and Mss11 to

UAS1-2 and leads to association of RNA polymerase II with the promoter (Kim, T.S. *et al.* 2004; Barrales, R.R. *et al.* 2008; Vinod, P.K.U. and Venkatesh, K.V. 2008).

Apart from this remodelling unit, the Rpd3L histone deacetylase complex including the members Pho23, Sap30, Rxt2, Sds3, Rpd3, Sin3, Dep1, Ash1, Ume1, Cte6, Rxt3 and Ume6 is involved in regulation of *ScFLO11* expression as well (Barrales, R.R. *et al.* 2008). The transcriptional activator Mss11 plays a large role in activation of *FLO11* within the MAPK pathway, too, whereas the Ras-dependent transcription factor Msn1 acts MAPK independent. Mss11 seems to be a key component in a complex that assists transcription factors in their regulation, and it forms complexes with Flo8 via their N-terminal LisH domains (Gagiano, M. *et al.* 1999; Gagiano, M. *et al.* 2003; Gerlitz G. *et al.* 2005). Deletion of *MSS11* results in complete absence of pseudohyphal growth, that cannot be suppressed by overexpressing Ste12 and Msn1 (Gagiano, M. *et al.* 1999). *MSS11* and *MSN1* can both be found in the genome of *A. gossypii* Whereas Mss11 acts downstream of Flo8 in *S. cerevisiae* (van Dyk, D. *et al.* 2005), it seems to be located upstream of Flo8 in *C. albicans* (Su, C. *et al.* 2009).

In *Candida albicans* overexpression of Swi/Snf bypasses the requirement of Ste12, Tec1 and Flo8 in invasive growth. In this organism Flo8 has a dual role as activator at high temperature and as repressor at low temperature (Su, C. *et al*, 2009). *FLO8* expression is repressed by Sfl1 in *S. cerevisiae* as well as in *C. albicans* (Kim, T.S. *et al.* 2004; Bauer, J. and Wendland, J. 2007).

The transcription factor Ste12 has a major role in filamentous growth but also in mating response in *S. cerevisiae* (Fields, S. and Herskowitz, I. 1985; Liu, H. *et al.* 1993). Multiple pheromone response elements (PREs; TGAAAC(A/G)) enable binding of the factor as a homomultimer or heterodimer with Mcm1 to promoters of mating genes (Kronstad, J.W. *et al.* 1987; Dolan, J.W. *et al.* 1989; Errede, B. and Ammerer, G. 1989). Filamentation genes bear filamentation/invasion response elements (FRE) that consists of a PRE adjacent to a Tec1-binding site (TCS; CATTC (C/YY)) Ste12 can bind those in cooperation with Tec1 (Madhani, H.D. and Fink, G.R. 1997). Tec1 bears an evolutionary conserved DNA binding domain with the name TEA (TEF-1, Tec1, AbaA) or ATTS (AbaA, Tef-1, Tec1, Scalloped) motif at its N-terminal region (Adrianopulos, A. and Timberlake, W.E. 1991, 1994; Laloux, I. *et al.* 1990; Campbell, S. *et al.* 1992; Xiao, J.H. *et al.* 1991). Binding sites in mating gene promoters are occupied by a complex of Ste12/Dig1/Dig2, whereas filamentation gene promoters are bound by Ste12/Dig1/Tec1. By replacing Dig2 in the complex, Tec1 tethers Ste12 to this subset of

genes. This explains why many of these genes do not possess FREs but all contain TCS (Chou, S. *et al.* 2006). During pheromone response as well as during filamentous growth a MAPK cascade regulates Ste12 activation. The central MAPKs in the mating pathway are Kss1 and Fus3, which phosphorylate Dig1 and Dig2 leading to their inactivation and setting Ste12 free to act as a transcriptional activator (Cook, J.G. *et al.* 1996). Fus3 specifically phosphorylates Tec1 and triggers ubiquitin mediated degradation of this protein (Bao, M.Z. *et al.* 2004). During filamentous growth Kss1 is the necessary MAPK (Cook, J.G. *et al.* 1997). Pheromone presence promotes sumoylation of Ste12 and diminishes sumoylation of Tec1 that leads to its degradation, because the same site is accessible for ubiquitination. This ensures impairment of the mating and the filamentation pathway (Wang, Y. and Dohlman, H.G. 2006). Since Tec1 has a higher affinity for Ste12 than the inhibitor Dig2, the amount of Tec1 determines the ratio of the two complexes and facilitates either filamentation or mating, especially since Tec1 expression is regulated by PREs itself (Chou, S. *et al.* 2006; Madhani, H.D. and Fink, G.R. 1997). Ste12 alone or in combination with Tec1 is necessary to drive expression of *TEC1*. One Tec1-binding site is sufficient for initiation of filamentation gene transcription, and Tec1 can mediate gene expression alone by binding to Tec1 binding sites with its C-terminal part (TCS control), whereas orientation of TCS is not important (Köhler, T. *et al.* 2002).

AbaA of *A. nidulans* binds to AbaA-responsive elements that are identical to TCS elements. It activates conidiophore development independently of the Ste12 similar protein SteA. This makes AbaA a regulator of asexual whereas SteA is a regulator of sexual development in this organism (Vallim, M.A. *et al.* 2000). In *C. albicans* serum-induced hyphal growth and virulence are primarily regulated by CaTec1 independently of CaCph1, which is a Ste12-like protein. CaCph1 regulates filamentous growth in response to nitrogen starvation (Liu, H. *et al.* 1994; Schweizer, A. *et al.* 2000).

2.3.2. Involvement of the cAMP-PKA pathway in adhesin expression

The cAMP/PKA pathway in *S. cerevisiae* has two triggers. The activity of the adenylate cyclase Cyr1 is increased by intracellular phosphorylation of glucose and Ras2 activation through extracellular glucose sensing via Gpr-Gpa2 (Rolland, F. *et al.* 2000). A G-protein coupled glucose and sucrose receptor system with the receptor Gpr1 and Gpa2 as the Gá protein stimulate Cyr1 activity after activation through Mep2. Gpr1 has

a dual receptor function since it senses glucose and structural related sugars, and its transcription is induced by low nitrogen levels (Xue, Y. *et al.* 1998; Lorenz, M.C. *et al.* 2000 b). A high cAMP level activates protein kinase A (PKA) which leads to dissociation of the Bcy1 regulatory subunits from the catalytic subunits Tpk. Tpk2 phosphorylates Sfl1, which leads to its inactivation by preventing dimerisation and DNA binding. It phosphorylates Flo8 as well, which becomes active and stimulates gene expression together with Mss11 (Gagiano, M. *et al.* 2003; reviewed in Gancedo, J.M. 2001; Sengupta, N. *et al.* 2007). The sequence TTTG(C/G)-n-(G/C)CAAA (n=97) is considered as the binding site for Flo8 and Mss11 (Bester, M.C. *et al.* 2006). In the absence of Flo8 the *ICR1* ncRNA is transcribed upstream of the *FLO11* ORF and represses *FLO11* transcription in cis by blocking access to general transcription factors. Flo8 initiates transcription of *PWR1* ncRNA from the complementary strand, which interferes with *ICR1* and therefore leads to transcription of *FLO11*. The histone deacetylase Rpd3L is strongly involved, since its binding upstream the *FLO11* ORF could block Sfl1 binding and therefore repress *ICR1* expression (Bumgarner, S.L. *et al.* 2009).

2.3.3. Adhesin expression regulated by the main glucose repression pathway and other factors

The main glucose repression pathway is based on intracellular glucose phosphorylation by hexokinases after uptake via hexose transporters in *S. cerevisiae*.The result of the phosphorylation or depletion of AMP inactivates the central Snf1 protein kinase. In turn Mig1, Nrg1, Nrg2 and Sfl1 bind to the promoter of *FLO11* under recruitment of repressor proteins Tup1 and Ssn6 leading to *FLO11* repression. At low glucose levels Snf1 phosphorylates Mig1, which leads to its localization in the cytoplasm and derepression of *FLO11* (Wilson, W.A. *et al.* 1996; Treitel, M.A. and Carlson, A. 1995, Kuchin, S. *et al.* 2002). Another target of Snf1 is Msn1 that has been described to have an important role in filamentous growth regulation (Cullen, P.J. and Sprague, G.F.Jr. 2000).

Independently from the MAPK and the cAMP pathway, *ScFLO11*expression can be positively regulated by Phd1, Swi5 and Ash1 (Gimeno, C.J. and Fink, G.R. 1994; Chandarlapaty, S. and Errede, B. 1998; Pan, X. and Heitman, J. 2000). It has been documented that a double mutation of *STE12*-and *PHD1*-like genes causes complete

loss of filaments in *S. cerevisiae* and *C. albicans* whereas single mutations do not (Lo, H.J. *et al.* 1997). The Cdc28-Cln2 complex has an impact on phosphorylation of Ste20 and therefore on regulation of filamentous growth, too (Wu, C. *et al.* 1998). Cln1 and Cln2 are important for the transcription of *FLO11* and pseudohyphal growth in *S. cerevisiae*. Grr1, the F-box protein in the SCF ubiquitin ligase complex, is involved in regulation of Cln1 and Cln2 in response to glucose- and amino acid availability. Low nitrogen levels result in downregulation of Cln3, a negative regulator of *FLO11* transcription. All three cyclins work independently from the Ste12-MAPK pathway. Further a Cln1/2-like G1 cyclin is involved in hyphal formation of *C. albicans* in certain media (Barral, Y. *et al.* 1995; Loeb, J.D.J. *et al.* 1999a, b).

The filamentous growth of *S. cerevisiae* in response to nutritional deprivation requires Flo8 protein in order to transcribe *FLO11*. It has been shown that *FLO8* mRNA contains an IRES (internal ribosome entry site) that promotes cap-independent translation with the help of eiF4G (Gilbert, W.V. *et al.* 2007).

During amino acid starvation of *S. cerevisiae*, Ste12 is not involved in *FLO11* expression, whereas the transcription factor Gcn4 recruits the Swi/Snf chromatin remodelling complex that enables Flo8 binding (Braus, G.H. *et al.* 2003; Yoon, S. *et al.* 2003). Amino acid starvation is sensed via the kinase Gcn2 that phosphorylates the translation initiation factor eIF2. This leads to minimising of Cap-dependent translation and preferred translational activation of specific mRNAs such as *GCN4* as part of the general control of amino acid biosynthesis (GAAC) (Hinnebusch, A.G. 2005).

Nitrogen limited conditions induce the *ScTOR* protein kinase, which together with Tap42 and Sit4 induce NCR (nitrogen catabolite repression) genes and pseudohyphal growth (DiComo, C.J. and Arndt, K.T. 1996; Cutler, N.S. *et al.* 2001).

Rim101 is the receptor in the pH-response pathway, which leads in acidic environments to repression of transcriptional suppressors like Nrg1 and Smp1 and therefore to expression of *ScFLO11* (Lamb, T.M. and Mitchell, A.P. 2003).

Hsl1 and Hsl7 interact with the septin ring and inhibit the regulator of the cell cycle, Swe1 (McMillan, J.N. *et al.* 1999). The interaction of Hsl7 with Ste20 prevents filamentous growth in yeast (Fujita, T. *et al.* 1999).

Levels of Ste12 and Gpa2 are increased during filamentous growth of *S. cerevisiae* by elevated translation of existing mRNA depending on *CAF20*, which encode an eIF4E inhibitor and Dhh1, an mRNA decapping activator (Park, Y.U. *et al.* 2006).

ScFig2, which is expressed upon pheromone stimulation in mating conditions, is necessary for cell fusion, agglutination and cell integrity during mating (Zhang, M. *et al.* 2002). A MAPK cascade with G-protein coupled receptors Ste2 or Ste3 is involved in its expression. Upon pheromone stimulation the inhibitory Gá subunit Gpa1 releases the Gâã subunits, which associate with the scaffold protein Ste5 and the protein kinase Ste20. The MAPK cascade, consisting of Ste11, Ste7 and a complex of Fus3 and Kss1, becomes activated and leads to derepression of the Tec1/Ste12 complex (reviewed in Gagiano, M. *et al.* 2002). Haploid cells of MATa mating type can be induced to grow filamentously by exposure to low dose of á-factor. This growth is independent of Tec1 and Flo11, and it requires Fig2 (Erdman, S. and Snyder, M. 2001; Guo, B. *et al.* 2000). The pheromone induced adhesins Aga1, Aga2, Agá1 and Fig2 show different interactions, such as between Aga1 and Aga2, Aga2 and Agá1, Aga1 and Fig2 and between Fig2 and Fig2. The latter two are less important and they are mediated by WCPL and CX_4C domains, which are present in multiple copies at the C-terminus of the proteins (Huang, G. *et al.* 2009).

The HOG pathway in *S. cerevisiae* becomes activated under osmotic stress conditions, and it shares components with the mating and the filamentous growth pathway. Specificity of the HOG pathway is maintained by phosphorylation of a protein by cytoplasmic Hog1, which binds to Tec1 in the nucleus and prevents its DNA binding (Shock, T.R. *et al.* 2009).

3. Materials and methods

3.1. Work with DNA

3.1.1. PCR reaction

All gene maps that were used as theoretical basis, were generated using Lasergene software version 7 (DNASTAR). 200 ng of genomic DNA, 5 µl 10 x PCR buffer including 15 mM $MgCl_2$ (Genaxxon, Ulm, Germany), 5 µl 25 mM $MgCl_2$ (Genaxxon), 5 µl 2 pM reaction primer each, 5 µl 2 mM dNTPs (Genaxxon), 0.2 µl 0.5 U Taq DNA polymerase (Genaxxon) and 20 µl water were mixed in a 50 µl reaction. The initial denaturation step contained incubation at 94°C for 4 min. This was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and synthesis at 72°C for 1 min. The final synthesis step at 72°C for 5 min terminated the reaction. The annealing temperature and synthesis time differed depending on melting temperature of the primer and fragment length. Used PCR machines were Mastercycler PCR machine (Eppendorf AG, Hamburg, Germany), Primus PCR machine (MWG Biotech, Ebersberg, Germany) or Primus 25 advanced PCR machine (peqlab, Erlangen, Germany).

To analyse the presence of plasmids or marker genes in *Ashbya gossypii*, *Saccharomyces cerevisiae* or *Escherichia coli* on a colony level, colony PCRs were performed. For this purpose components of the PCR reaction including 25 µl water instead of 20 µl were premixed and put in a PCR vial. A piece of the colony was transferred into the mixture, and the PCR reaction was carried out as described using a Speedcycler (Analytic Jena AG, Jena, Germany).
Oligonucleotidprimer that were used in this work were purchased from biomers.net GmbH, Ulm, Germany and are shown in table1.

Oligonucleotidprimer that were used in this work

primer	primer sequence 5-3'
AgFLO5-1	
S1-AgFLO5-1	CTTGTAACTCTCACGTCAAGAAGGAATACCTGCAAT
	CGCTGTAACTAATATTGACAAGGGTTAAGgaagcttcgta
	cgctgcaggtc
S2-AgFLO5-1	GGAGTATATTAAGAACACAATTGGGATTCATTGGGC
	ACTACTAAACTAGGAAGTCAGCtctgatatcatcgatgaattcgag
G1-AgFLO5-1	GCTCAATTTCAACGTGAGCAGGTG
G4-AgFLO5-1	GTCGCCGTCCGGTGATTAGTTAAGC
I1-AgFLO5-1	AGAGGAATCAACAGTAACCAGCCCTTCG
I2-AgFLO5-1	GGTAGATGGTCTCGGTGGTTGCG
I3-AgFLO5-1	CAGTGCAGTCTGGTCGAGAG
I4-AgFLO5-1	CGATACCATCAGTACCGGTCAC

I5-AgFLO5-1	CGCGACAACGACTCATCCAT
I6-AgFLO5-1	AGCCGTCACGACGACTACGAG
I1-new-AgFLO5-1	CGACCGAGTTAATCGCGGTG
I2-new-AgFLO5-1	GTTCCATCGTATGGATGAGTC
G1-AgFLO5-1 SN	CACGTGATGCTTCTGAACTG
5'-AgFLO5-1a (EcoRI)	CTTCTTgaattcCTTCCATCTTGGGAATTATG
5'-AgFLO5-1b	gacctgcagcgtacgaagcttcCTTAACCCTTGTCAATATTAG
3'-AgFLO5-1a	cgatactaacgccgccatccagGCTGACTTCCTAGTTTAGTAG
3'-AgFLO5-1b (XhoI)	ATTATTctcgagCTTCGGTTCCATGCTACGATC
G4-AgFLO5-1 SN	CGGTCTTAGTTACGTACTG
KpnI-5'prom-AgFLO5-1	TTTCTAggtaccCTGCTGTTTCCGGGTCCTGTC
XhoI-3'prom-AgFLO5-1	TCTTTTctcgagCTTAACCCTTGTCAATATTAGTTACAG CG
AgFLO5-2	
S1-AgFLO5-2	TTGCCTTTCTATCAGTTTTAAAATTAGTCATTTGAGA AAGTCTGTGAAGGCCTCGgaagcttcgtacgctgcaggtc
S2-AgFLO5-2	GCAGATATATAAGGTGTGACGCGGGCACAGCGACA GCGCATTCGACAGGGCAAAGAtctgatatcatcgatgaattcgag
G1-AgFLO5-2	CGGCTATATTCCTAGAGAGGAAACAG
G4-AgFLO5-2	CACGGCTGCGGAATTGGGTC
I1-AgFLO5-2	CACAGGTTGCTCTGAAGTGCCAG
I2-AgFLO5-2	GAGTCTCTAACAACGGTCACCAGC
G1-AgFLO5-2 SN	GCAGGATCAATCGATAACC
5'-AgFLO5-2a (EcoRI)	CTTCTTgaattcGCTTAACTAATCACCGGAC
5'-AgFLO5-2b	gacctgcagcgtacgaagcttcAAACGAGGCCTTCACAGAC
3'-AgFLO5-2a	cgatactaacgccgccatccagTCTTTGCCCTGTCGAATGCG
3'-AgFLO5-2b (XhoI)	ATTATTctcgagCTCACTGTCCCACAAGCATG
G4-AgFLO5-2 SN	GCGATTCTAAAGCTGTTGCC
KpnI-5'prom-AgFLO5-2	TTTCTAggtaccGCTGCGATTCTAAAGCTGTTGCCG
XhoI-3'prom-AgFLO5-2	TCTTTTctcgagTCTTTGCCCTGTCGAATGCGCTG
AgFLO8	
S1-AgFLO8	CATCACCAGAGCGGCAGGAGTTGCAGTAGCCTCAG GAGGACCGGACGCTACAGAACTGAAGCCAGgaagcttc gtacgctgcaggtc
S2-AgFLO8	CGGCATCCTCCTGATCGAACTGGAAGGGCACATCGC CGCTTAGGGTAGGGTCGTCGTGGAtctgatatcatcgatgaattc gag
G1-AgFLO8	GCGAACACGTTTATGTCGCCTC
G4-AgFLO8	GTTGGCGGTGTCGGTGCTG
I1-AgFLO8	GCGTCGTACTGCGTCCACGCAG
I2-AgFLO8	GAGCCTGCTGTTGAGCGTGCTG
I3-AgFLO8	GAGTGGTGGCAGATCTTCTGGG
I4-AgFLO8	GCGGTAGTTATGCAAGCCGTC
5'-AgFLO8	CCGCACGTGACGGGAGGACTGC
3'-AgFLO8	CTACTGGCACATGATCTCTACATCAG
I5-AgFLO8	ATCAACAGGTATCGTATCCAC
I6-AgFLO8	GACAGTTTCCATCAGGCTATC
G1-AgFLO8 SN	GTCAGGGTGTTATCATTCG
5'-AgFLO8a (EcoRI)	CTTCTTgaattcCTCGCCTGATTGTGCTACC
5'-AgFLO8b	gacctgcagcgtacgaagcttcCTGGCTTCAGTTCTGTAGCG
3'-AgFLO8a	cgatactaacgccgccatccagCGCAGTTCTCCGTCACACA
3'-AgFLO8b (XhoI)	ATTATTctcgagGTACGTAGACAGGTGCGAG
G4-AgFLO8 SN	GTTAGCCGGATTGTTGATCG
KpnI-5'prom-AgFLO8	TTTCTAggtaccCCGCCCAGTGTATAAGCATCGGC
XhoI-3'prom-AgFLO8	TCTTTTctcgagCTGGCTTCAGTTCTGTAGCGTCC
AgFLO11	
S1-AgFLO11	CCTCAGTTTCGTCCCTCGAATAGCCAATCCAGCCAG GACACTTCAGAATCgaagcttcgtacgctgcaggtc
S2-AgFLO11	CAAAGACCGAGATGTAAGTCGAAGGAGCTGTCGCT GAGGAAGAGTCCCGCGATGGATAACtctgatatcatcgatgaa ttcgag

G1-AgFLO11	GGCTCCTTCAAAGCCCCGATGC
G4-AgFLO11	CAAGGAAGCTACGACAGTAGTG
I1-AgFLO11	CCAGGTACAGCGAACCACG
I2-AgFLO11	GTACGCGGGCGAGTCCTTC
G1-AgFLO11 SN	CAGACTATCAAGACTCTCG
5'-AgFLO11a (EcoRI)	CTTCTTgaattcGCTGAGCTACCGTCATATC
5'-AgFLO11b	gacctgcagcgtacgaagcttcGATTCTGAAGTGTCCTGGC
3'-AgFLO11a	cgatactaacgccgccatccagGGGTTAAACCCCGGATACC
3'-AgFLO11b (XhoI)	ATTATTctcgagCGCGAGGTCATCACTATTAC
G4-AgFLO11 SN	GACATCTGACACTCTGTGC
KpnI-5'prom-AgFLO11	TTTCTAggtaccCTACCCGGGCTGAAGCCCG
XhoI-3'prom-AgFLO11	TCTTTTctcgagGATTCTGAAGTGTCCTGGCTGGATTGG
AgSFL1	
S1-AgSFL1	GGTTCGACGCGCCAACTACAGGCGACGTCGACGGA GCAGCATAACTTGGAGgaagcttcgtacgctgcaggtc
S2-AgSFL1	AGTGTATAGGTAACCTGAATATATTCGGAAGTGACA TACAACTGGTATTTTGCTAGTTGCtctgatatcatcgatgaattcg ag
G1-AgSFL1	GAGGTCTTTTATCCTGTATCCAGCG
G4-AgSFL1	TGGATTGGGAGATGCTTGGTAGCAG
I1-AgSFL1	GAGCGTGATCACACTCCCACATCTG
I2-AgSFL1	CTAAAGATGGTACCTGCATTGCTGC
G1-AgSFL1 SN	CTGCTTTAGATGCTGTCGAG
5'-AgSFL1a (EcoRI)	CTTCTTgaattcCACTAGAGCATCGGAGTAAC
5'-AgSFL1b	gacctgcagcgtacgaagcttcCTCCAAGTTATGCTGCTCCG
3'-AgSFL1a	cgatactaacgccgccatccagTATACGGTTAAGGAACTTCC
3'-AgSFL1b (XhoI)	ATTATTctcgagCGGTATACAACTGTAGGAT
G4-AgSFL1 SN	GACCCCTCAATGTTAACGTG
KpnI-5'prom-AgSFL1	TTTCTAggtaccACTATAAGTCGCCAGTCTTGGCGTGC
XhoI-3'prom-AgSFL1	TCTTTTctcgagCTCCAAGTTATGCTGCTCCGTCGAC
5'-AgSFL1-SalI	TCTGTGgtcgacATGGCGCAATTGGAAGATCCG
3'-AgSFL1-SacI	ATTATTgagctCGGAAGTGACATACAACTGG
AgSFL1 rev (EcoRI)	ATTATTgaattcGTGGAACCCGTACATGTTC
S1-GFP-AgSFL1	CTGCGACCAGCAAAAGCAGAGGTTTGTACGCACTCT TAAATCACGATACTggggccggtgcaggcgctgga
S2-GFP-AgSFL1	CGAACAGATGAACCGAGAAATATCTATCTCAATTAA CAAGCATTAGCCACagggacctggcacggagc
5'-AgSFL1	CTGGTACTGCCTCCACCTCTTCG
AgFIG2	
S1-AgFIG2	CACGCACGATGCGTGTCCGAATGGAACCCTCCAGGA AGGCAGTCAGGATTAATCCAAgaagcttcgtacgctgcaggtc
S2-AgFIG2	AGTTAATTATGTGTGCGCGTGCGCGCAGAATTGATT ACACAAATTGACCTGGAGCGAGTCtctgatatcatcgatgaattc gag
G1-AgFIG2	CGTTGCCTCTAACGTTCCTCTTC
G4-AgFIG2	GGTGATCCAAGCGGCGTTGCAGG
I1-AgFIG2	GAAGGTGACCGCACCAGAC
I2-AgFIG2	GACAGAGCCGTCGATGGTG
I3-AgFIG2	CCACTGCTCCAGCTCCAGAG
I4-AgFIG2	GTGCAGCGACGATTGCACCTG
G1-AgFIG2 SN	CAGCATCCACAGTTGCATTC
5'-AgFIG2a (EcoRI)	CTTCTTgaattcGAAGCTCTACCACTGGATG
5'-AgFIG2b	gacctgcagcgtacgaagcttcGATTAATCCTGACTGCCTTC
3'-AgFIG2a	cgatactaacgccgccatccagCTCGCTCCAGGTCAATTTG
3'-AgFIG2b (SacI)	AGGATTTAgagctcCAGAACCGCGTGTTCATTGC
G4-AgFIG2 SN	CAAGCGTTATCGTGAAGAAC
AgTEC1	
S1-AgTec1	CGAATTTGATCCGGGCTGTGAGCAACGCAGCAAAA GAGCGGGATTTGGCGAAAGCGCAGCGgaagcttcgtacgct gcaggtc
S2-AgTec1	TGAGCTGCTCTCTTGCTCAGTATTTTCTTCTTTAAAC

	AGGATATATTATGCCCACTTCATGtctgatatcatcgatgaattcgag
G1-AgTEC1	GATCGCTGGTTGTGGTTGGAAGCAG
G4-AgTEC1	CACAGTAAGGCAACTGTATACTCGC
I1-AgTEC1	GAGTTCGAGAAGGAGCTGCTGAACCTC
I2-AgTEC2	GTGGTTGTTGCTGAAATAGCTCCTC
KpnI-5'prom-AgFIG2	TTTCTAggtaccATATGTAAAAGCCTGAATTCGCAGGACACGG
XhoI-3'prom-AgFIG2	TCTTTTctcgagTTGGATTAATCCTGACTGCCTTCCTGGAGGG
AgSTE12	
G1-AgSTE12	GTAGCTGTCACCAGTGCGAG
G4-AgSTE12	GACATTCTGGAAGTTTACTGGC
ScSFL1	
5'ScSFL1-PstI	CTTGActgcagATGAGTGAAGAGGAAACGGTCTCAGC
3'ScSFL1-XbaI	CTTGAtctagaGATACGAATTGAAGGGAGGTAGG
kanMX	
S1-annealing region	
kanMX	GAAGCTTCGTACGCTGCAGGTC
new S2-annealing region	
kanMX	CTGGATGGCGGCGTTAGTATCG
GEN3	
G2new (G418)	GCCAGTTTAGTCTGACCATC
G3new (G418)	TCGCAGACCGATACCAGG
G2 (G418)	GTTTAGTCTGACCATCTCATCTG
G3 (G418)	TCGCAGACCGATACCAGGATC
NAT1	
AgNAT1up1	CGGCGTCCCCCGGGACACTGG
AgNAT1do1	CCGCCTCGGACGGCGAGCGGC
StlacZ	
LACZ-intern-up	CCATTAAGAGATTGTCTTAACTTCATCTC
AgTEF1	
5'AgTEFp KpnI	TATCGGggtaccGATCTGTTTAGCTTGCCTCGTCC
AgTEF-prom	AGGATTTGCCACTGAGGTTCTTC
ScTEF1	
ScTEFp-up1	GGGTAATTTGTCGCGGTCTGGG
ScTEFt-down1	GCCCATCAGATTGATGTCCTCC
ScTEF2p-down	TCAAGCGTAGGCGCTTCC
GFP	
XFP-fusion seq primer	CATAACCTTCGGGCATGGCACTC
pSK+, pGEM	
M13 uni	CGACGTTGTAAAACGACGGCCAGTG
M13 rev	CACACAGGAAACAGCTATGACC

Table1. Shown are oligonucleotidprimer used in this work. All sequences are written from 5‘ to 3‘. Lower case sequences correspond to annealing regions for the amplification of pFA-cassettes or additions for restriction sites (underlined).

3.1.2. Gene deletions via PCR based gene targeting

The method is based on Wendland, J. *et al.* (2000).

Antibiotic resistance genes were amplified from a plasmid using S1 and S2 primers containing a 50 bp homologous region to the 5’ and 3’ untranslated region of the gene, respectively, and a 22 bp homologous region to the marker gene. The PCR conditions included an initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 93°C for

1 min, annealing at 52°C for 1 min and synthesis at 72°C for 2 min 30 s and a final synthesis step at 72°C for 5 min. 10 µl of the PCR product was examined on a 0.83% analytical agarose gel, the rest was mixed with 1/10 vol 3 M sodiumacetate (Merck) and 1 vol isopropanol (Niels Peter Mark, Valby, Denmark) and centrifuged at 13000 rpm for 25 min. After discarding the supernatant, 500 µl 70% ethanol (Kemetyl AS Koge, Denmark) were added followed by centrifugation at 13000 rpm for 5 min. The DNA was dried and dissolved in 50 µl water and then used for transformation in *Ashbya gossypii* (Fig.2).

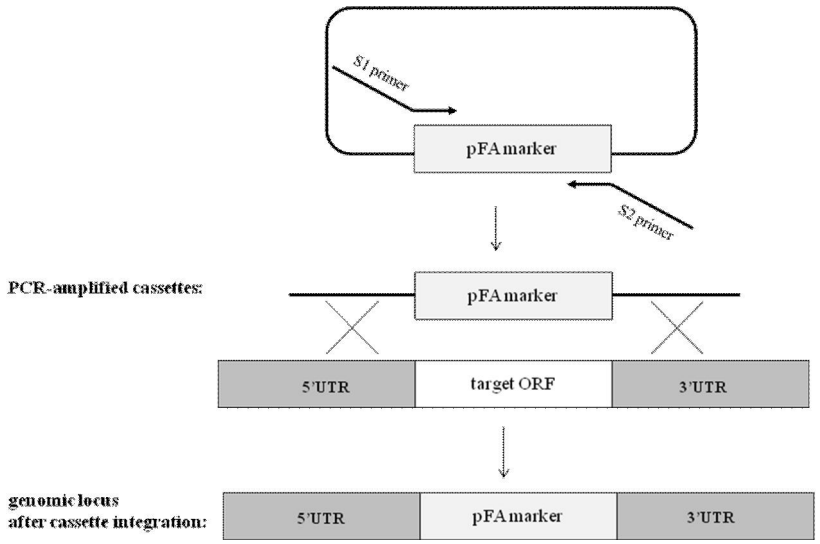


Figure 2. Gene deletion scheme based on PCR amplification of marker genes using S1 and S2 primers with 50 bp homology region to either 5'- or 3'-UTR of the target gene.

3.1.3. Gene deletions with deletion cassettes

The method is based on Noble, S.M. and Johnson, A.D. (2005).

The *kanMX* resistance marker was amplified from a plasmid using the primers "S1-annealing region kanMX" and "new-S2-annealing region kanMX". 5' and 3' untranslated regions of about 250-350 bp of the genes were amplified from *Ashbya* genomic DNA with primers 5'a and 5'b for the 5' flank and 3'a and 3'b for the 3'flank. The used primers introduced an *Eco*RI restriction site at the 5' site of the 5' flank and an *Xho*I site at the 3' site of the 3' flank. The PCR conditions included an initial step at 94°C for 4 min followed by 35 cycles of 93°C for 1 min, 55°C for 1 min and 72°C for 1 min. The final synthesis was carried out at 72°C for 5 min. At the 3' site of the 5' flank and at the 5' site of the 3' flank, homologous regions to the *kanMX* gene were introduced with the used primers. The 5'a primer of the 5' flank, the 3'b primer of the

3' flank were used together with the 5' and 3' flank of the genes and the *kanMX* marker as templates in a following PCR. A fusion product consisting of 5' flank of the gene, *kanMX* marker gene and 3' flank of the gene was created. The PCR conditions included an initial step at 94°C for 4 min followed by 35 cycles of 93°C for 1 min, 55°C for 1 min and 72°C for 3 min. The final synthesis was carried out at 72°C for 5 min. Using the introduced restriction sites, the cassette was digested with *Eco*RI and *Xho*I as was the vector pSK+. Cassette and vector were ligated, propagated in *E. coli* DH5á and purified. Prior transformation of *A. gossypii* the cassette was released from the vector backbone by digest with *Eco*RI and *Xho*I (Fig.3).

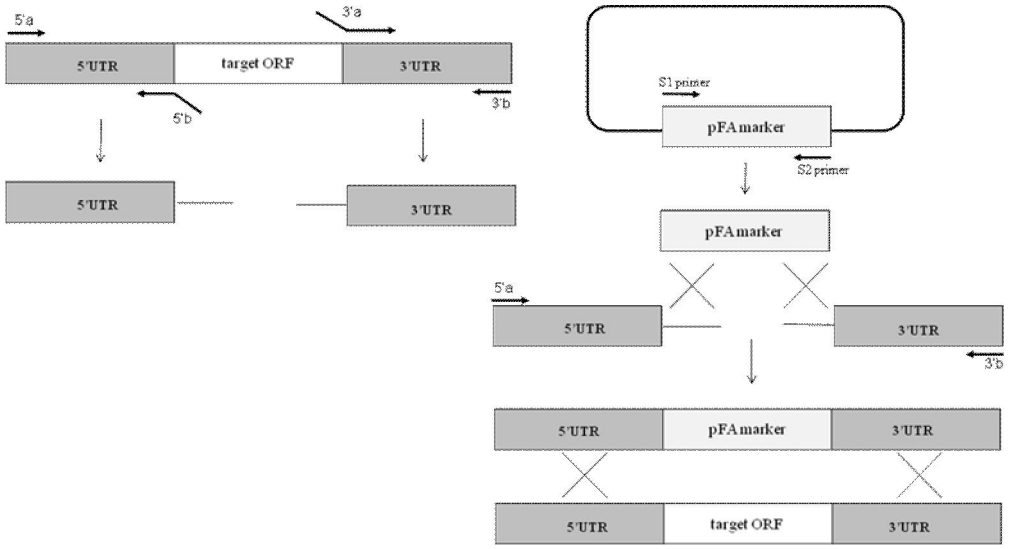


Figure 3. Gene deletion scheme based on deletion cassettes.

3.1.4. Verification PCR on DNA from *Ashbya* hetero- and homokaryons

To analyse the presence of the marker gene, three sets of primers were used in three PCR reactions of 50 µl each. The first set contained a primer that binds in the 5' untranslated region of the gene of interest, named G1, and one that binds in the 5' region of the marker gene, named G2, respectively. The second set contained a primer that binds in the 3' region of the resistance gene, named G3, and another one that binds in the 3' untranslated region of the gene, named G4. The third set contained primers, named I1 and I2, that amplify a region inside the gene of interest and lead only to a PCR fragment if at least one copy of the gene is still present for example in a heterokaryon. A homokaryon therefore should only lead to PCR fragments with primers G1-G2 and G3-G4 but not with I1-I2 (Fig.4). The PCR conditions for verification PCRs contained an initial melting step at 94°C for 4 min followed by 35 cycles of repeated melting at 93°C

for 1 min, annealing at 52°C for 1 min and synthesis at 72°C for 1 min. The final synthesis occurred at 72°C for 5 min. Annealing temperatures and synthesis time differed, depending on melting temperature of the primers and fragment length.

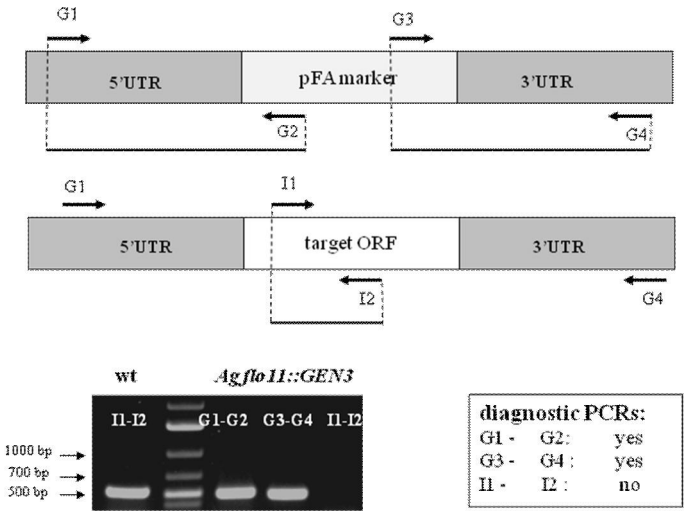


Figure 4. Verification PCR of gene deletions. Primers G1-G2 and G3-G4 amplify a fragment if the pFA marker is correctly inserted. Internal primers I1-I2 amplify an internal part of the gene if at least one copy is still present in the genome. An example of a verification PCR in *Agflo11::GEN3*is shown on the bottom left and the expected fragments in a diagnostic PCR on DNA of a homokaryon on the bottom right.

3.1.5. Agarose gel electrophoresis of DNA

For the separation of PCR fragments or restriction digest products, 0.83% agarose (Roth, Karlsruhe, Germany) gels in 1 x TAE (TRIS-Acetic acid-EDTA) buffer were used. For the visualization of DNA in UV light, the gels contained 0.5 µg/ml ethidiumbromide (Sigma). DNA was mixed with 1/10 vol loading buffer containing 0.2% bromphenol blue, 0.2% xylencyanole, 0.2% orange G, 50% saccharose and 1 mM EDTA. O GeneRuler 1kb Plus DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany) or *Pst*I cleaved DNA of phageλ (MBI Fermentas) was used as a size standard. Visualization was done using Pharmacia Biotech ImageMaster VDS gel documentation system (Amersham Pharmacia Biotech Inc., New Jersey, USA).

1 l 50 x TAE consists of 242 g TRIS (J.T. Baker), 57 ml glacial acetic acid (Fluka), 100 ml 0.5 M EDTA (Sigma) and water up to 1 l.

3.1.6. Restriction digests of plasmids and inserts

Vector backbones and inserts were restriction digested in 150 µl reactions consisting of 50 µl DNA, 15 µl BSA, 15 µl 10 x digestion buffer (NEB), 3 µl enzyme (NEB) and 70 µl water. The reaction was incubated according to specifications of the enzyme suppliers.

3.1.7. Analytical restriction digest of plasmid preparations

Plasmid preparations were restriction digested in 20 µl reactions containing 2 µl DNA, 2 µl 10 x digestion buffer, 2 µl BSA, 0.3 µl enzyme and 14 µl water. The reaction was incubated according to specifications of the enzyme suppliers.

3.1.8. Purification of PCR- and digestion products

Products from a restriction digest were loaded on a 0.83% agarose gel in 1 x TAE buffer and separated at 120 V, 40 mA for 45 min. DNA bands were localised using Macrovue UV-26 universal voltage table (Hoefer Inc., Massachusetts, USA), cut out and purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA).

3.1.9. Ligation of PCR products into vector backbones

Restriction digested and gel extracted vectors were dephosphorylated prior to ligation. For this purpose 45 µl of digested vector backbone were mixed with 5 µl 10 x SAP buffer (Promega) and 1 µl Shrimp Alkaline Phosphatase (1 U/µl, Promega) and incubated at 37°C for 15 min. The SAP was heat inactivated at 65°C for 15 min, and the reaction was centrifuged at 13000 rpm for 5 min with a Centrifuge 5415 C (Eppendorf) or Pico 17 centrifuge (Heraeus, Hanau, Germany).

For the ligation 3 µl of vector backbone, 7 µl of insert, 2 µ of 10 x ligation buffer (Fermentas), 0.2 µl of T4 DNA ligase (1 U/µl, Fermentas) and 8 µl of water were mixed and incubated at 16°C for a minimum of 3 hours.
For amplification of PCR products, pGEM Teasy (Promega) or pDrive PCR cloning kit (Qiagen, Hilden, Germany) was used according to manufacturer's instructions.
For construction of expression vectors, PCR products and vector backbones were restriction digested and gel purified prior to ligation.

3.1.10. Sequencing of DNA

Sequencing was done by Eurofins MWG Operon, Germany according to company specification.

3.2. Work with *Ashbya gossypii*

3.2.1. Spore isolation from *Ashbya*

200 ml Complete supplement mixture (CSM) containing 0.3 g/l myo-inositol (Sigma) were inoculated with 1 ml of a preculture of *Ashbya* mycelium and incubated in a baffled flask at 30°C, 180 rpm in an Innova 44 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) for maximum one week until abundant spore production occurred. The culture was centrifuged at 4000 rpm for 10 min in a Multifuge 1 S-R (Heraeus), and after removal of the supernatant, the mycelium was resuspended in 30 ml 1 x Tris-EDTA (TE) buffer containing 5 mg zymolyase (Seikagaku Corporation, Tokyo, Japan). The following incubation at 37°C with gentle shaking for maximal 60 rpm on a GyroMini nutating mixer (Labnet International Inc., Edison, USA) was used for spore release. This was extended until all cell walls had been degraded.
After centrifugation at 4000 rpm for 10 min and removing of the supernatant, the spores were resuspended in 5-10 ml spore buffer and transferred in 1.5 ml vials. The spores were centrifugated at 5000 rpm for 5 min, and after removal of the supernatant resuspended in 1 ml spore buffer. After a following centrifugation at 5000 rpm for 5 min and removal of the supernatant, the spore pellet was resuspended in 1 ml spore buffer containing 25% glycerol and stored at –80°C.

1 l CSM consists of 20 g glucose (AppliChem GmbH, Darmstadt, Germany), 1.7 g YNB without amino acids and without ammonium sulphate (BIO 101 Systems, Q-BIO gene, Solon, OH, USA), 0.69 g CSM (BIO 101 Systems, Q-BIO gene) 1 g sterile filtrated asparagin (Sigma), optional 20 g agar (Roth) and water up to 1 l.
10 x TE contains 100 mM Tris, 10 mM EDTA (Sigma) and distilled water.
Ashbya spore buffer contains 0.03% Triton X100 (Pharmacia Biotech) in water.

3.2.2. Electroporation of *Ashbya gossypii*

The method is based on Wendland, J. *et al.* (2000).
200 ml of *Ashbya* full medium (AFM) were inoculated with a spore suspension and incubated in a baffled flask at 30°C for 18 h while shaking at 180 rpm. After harvesting by vacuum filtration through Whatman filter (Schleicher & Schuell, Dassel, Germany) and washing with water, the mycelium was resuspended in 40 ml 50 mM potassium phosphate buffer, pH 7.5, containing 25 mM DTT (Sigma) and incubated at 30°C for 30 min while shaking at 60 rpm. The mycelium was harvested by vacuum filtration, washed once with cold STM buffer, harvested again by vacuum filtration and resuspended in enough STM buffer to obtain a viscous solution. For the electroporation a maximum of 50 µl DNA-solution (5 - 20 µg) was mixed with 200 µl mycelium suspension and transferred in pre-chilled 4 mm cuvettes (peqlab). After application of a pulse of 1.5 kV, 100 Ω, 25 µF in the Equibio electroporator (Easyject Prima, Wolf lab, Pocklington, UK), 1 ml AFM was added to the transformation mixture. The liquid was plated on AFM plates and incubated at 30°C for 6 h. An overlay of 8 ml water containing 0.5% agarose and selective antibiotics was added, and after one to two days at 30°C transformants appeared. Primary transformants were tested for marker integration via PCR. Primary transformants are heterokaryons, since only some nuclei integrated the resistance marker. They were inoculated in selective CSM, and spores were isolated. Spores were incubated in selective AFM at 30°C, 180 rpm for 16 h. The isolate *Eremothecium gossypii* was proceeded in the same way.
Generated and used transformants are listed in table 2 and table 3.

1 l AFM medium contains 10 g casein peptone (Carl Roth GmbH & Co, Karlsruhe, Germany), 10 g yeast extract (Becton, Dickinson and Company. Sparks, MD, USA), 20 g glucose, 1 g filter sterilized myo–inositol that was added after autoclaving, optional 20 g agar and water up to 1 l. Optional after autoclaving were added geneticin sulphate (G418, Genaxxon) up to a final concentration of 200 µg/ml or clonNAT (kindly provided by HKI, Jena, Germany) up to a final concentration of 100 µg/ml.
For AFM with high sugar concentration 200 g glucose or fructose (Sigma), respectively, were used instead of 20 g glucose. The sugar was added from a filter sterilized 50% stock solution.
100 ml 1 M potassium phosphate buffer pH 7.5 contains 83.6 ml 1 M K_2HPO_4 (Merck, Darmstadt, Germany) and 16.4 ml 1 M KH_2PO_4 (Merck).

STM (Saccharose-TRIS-$MgCl_2$) buffer contains 270 mM saccharose (Merck), 10 mM TRIS and 1 mM $MgCl_2$ (Fluka) in water. The buffer was filter sterilized.

Generated and used *Ashbya gossypii* strains

strain	genotype	source
leu2	leu2	Mohr, Philippsen
AWA32a	leu2, ste12::KANMX	Walther
AWE84	leu2, ste4::KANMX	Wendland
AWE85	leu2, gpa1::KANMX	Wendland
AGA10	leu2, flo5-1::GEN3	this work
AGA11	leu2, flo5-2::NAT1	this work
AGA12	leu2, flo5-2::NAT1	this work
AGA13	leu2, flo5-1::GEN3	this work
AGA14	leu2, flo5-1-2::NAT1	this work
AGA15	leu2, flo5-1-2::NAT1	this work
AGA19	leu2, sfl1::NAT1	this work
AGA20	leu2, sfl1::NAT1	this work
AGA21	leu2, flo8::NAT1	this work
AGA23	leu2, tec1::GEN3	this work
AGA24	leu2, tec1::GEN3	this work
AGA25	leu2, tec1::GEN3	this work
AGA26	leu2, tec1::GEN3	this work
AGA27	leu2, flo8::NAT1	this work
AGA29	leu2, sfl1::NAT1	this work
AGA39	leu2, flo5-2::KANMX	this work
AGA40	leu2, flo5-2::KANMX	this work
AGA41	leu2, sfl1::KANMX	this work
AGA42	leu2, sfl1::KANMX	this work
AGA43	leu2, sfl1::KANMX	this work
AGA44	leu2, sfl1::KANMX	this work
AGA45	leu2, flo11::KANMX	this work
AGA46	leu2, flo11::KANMX	this work
AGA48	leu2, fig2::KANMX	this work
AGA49	leu2, fig2::KANMX	this work
AGA50	leu2, fig2::KANMX	this work
AGA51	leu2, fig2::KANMX	this work
AGA54	leu2, flo8::KANMX	this work
AGA55	leu2, flo8::KANMX	this work
AGA58	leu2, flo5-1::KANMX	this work
AGA59	leu2, flo5-1::KANMX	this work
AGA60	leu2, flo5-1-2::KANMX	this work
AGA61	leu2, flo5-1-2::KANMX	this work
AGA62	leu2, flo5-1-2::KANMX	this work
AGA63	leu2, flo11::KANMX	this work
AGA64	leu2, flo11::KANMX	this work
AGA65	leu2, flo8::KANMX	this work
AGA66	leu2, flo8::KANMX	this work
AGA67	leu2, flo11::KANMX	this work
AGA68	leu2, flo11::KANMX	this work
AGA69	leu2, pRS AgFLO5-1p lacZ NAT5	this work
AGA70	leu2, pRS AgFLO5-2p lacZ NAT5	this work
AGA71	leu2, pRS AgFLO8p lacZ NAT5	this work
AGA72	leu2, pRS AgFLO11p lacZ NAT5	this work
AGA73	leu2, pRS AgSFL1p lacZ NAT5	this work
AGA74	leu2, pRS AgFIG2p lacZ NAT5	this work
AGA75	leu2, pRS AgTEFp lacZ NAT5	this work
AGA76	leu2, flo8::KANMX, pRS AgFLO5-1p lacZ NAT5	this work
AGA77	leu2, flo8::KANMX, pRS AgFLO5-2p lacZ NAT5	this work

AGA78	leu2, flo8::KANMX, pRS AgFLO8p lacZ NAT5	this work
AGA79	leu2, flo8::KANMX, pRS AgFLO11p lacZ NAT5	this work
AGA80	leu2, flo8::KANMX, pRS AgSFL1p lacZ NAT5	this work
AGA81	leu2, flo8::KANMX, pRS AgFIG2p lacZ NAT5	this work
AGA82	leu2, flo8::KANMX, pRS AgTEFp lacZ NAT5	this work
AGA83	leu2, sfl1::KANMX, pRS AgFLO5-1p lacZ NAT5	this work
AGA84	leu2, sfl1::KANMX, pRS AgFLO5-2p lacZ NAT5	this work
AGA85	leu2, sfl1::KANMX, pRS AgFLO8p lacZ NAT5	this work
AGA86	leu2, sfl1::KANMX, pRS AgFLO11p lacZ NAT5	this work
AGA87	leu2, sfl1::KANMX, pRS AgSFL1p lacZ NAT5	this work
AGA88	leu2, sfl1::KANMX, pRS AgFIG2p lacZ NAT5	this work
AGA89	leu2, sfl1::KANMX, pRS AgTEFp lacZ NAT5	this work
AGA90	leu2, sfl1::KANMX, pRS AgSFL1p SFL1-GFP	this work
AGA91	leu2, tec1::GEN3, pRS AgFLO5-1p lacZ NAT5	this work
AGA92	leu2, tec1::GEN3, pRS AgFLO5-2p lacZ NAT5	this work
AGA93	leu2, tec1::GEN3, pRS AgFLO8p lacZ NAT5	this work
AGA94	leu2, tec1::GEN3, pRS AgFLO11p lacZ NAT5	this work
AGA95	leu2, tec1::GEN3, pRS AgSFL1p lacZ NAT5	this work
AGA96	leu2, tec1::GEN3, pRS AgFIG2p lacZ NAT5	this work
AGA97	leu2, tec1::GEN3, pRS AgTEFp lacZ NAT5	this work
AGA98	leu2, tec1::GEN3, pRS 418	this work
AGA99	leu2, fig2::KANMX, pRS AgFLO5-1p lacZ NAT5	this work
AGA100	leu2, fig2::KANMX, pRS AgFLO5-2p lacZ NAT5	this work
AGA101	leu2, fig2::KANMX, pRS AgFLO8p lacZ NAT5	this work
AGA102	leu2, fig2::KANMX, pRS AgFLO11p lacZ NAT5	this work
AGA103	leu2, fig2::KANMX, pRS AgSFL1p lacZ NAT5	this work
AGA104	leu2, fig2::KANMX, pRS AgFIG2p lacZ NAT5	this work
AGA105	leu2, fig2::KANMX, pRS AgTEFp lacZ NAT5	this work
AGA106	leu2, fig2::KANMX, pRS 418	this work
AGA107	leu2, ste12::KANMX, pRS AgFLO5-1p lacZ NAT5	this work
AGA108	leu2, ste12::KANMX, pRS AgFLO5-2p lacZ NAT5	this work
AGA109	leu2, ste12::KANMX, pRS AgFLO8p lacZ NAT5	this work
AGA110	leu2, ste12::KANMX, pRS AgFLO11p lacZ NAT5	this work
AGA111	leu2, ste12::KANMX, pRS AgSFL1p lacZ NAT5	this work
AGA112	leu2, ste12::KANMX, pRS AgFIG2lacZ NAT5	this work
AGA113	leu2, ste12::KANMX, pRS AgTEFp lacZ NAT5	this work
AGA114	leu2, ste12::KANMX, pRS 418	this work
AGA115	leu2, pRS AgCTS2p lacZ NAT5	this work
AGA116	leu2, flo8::KANMX, pRS AgCTS2p lacZ NAT5	this work
AGA117	leu2, sfl1::KANMX, pRS AgCTS2p lacZ NAT5	this work
AGA118	leu2, fig2::KANMX, pRS AgCTS2p lacZ NAT5	this work
AGA119	leu2, tec1::GEN3, pRS AgCTS2p lacZ NAT5	this work
AGA120	leu2, ste12::KANMX, pRS AgCTS2p lacZ NAT5	this work
AGA121	leu2, pRS AgRIB3p lacZ NAT5	this work
AGA122	leu2, ste12::KANMX, pRS AgRIB3p lacZ NAT5	this work
AGA130	leu2, pRS AgTEFp SFL1	this work
AGA131	leu2, pRS AgTEFp SFL1	this work
AGA132	leu2, pRS AgTEFp SFL1	this work
AGA133	leu2, pRS AgTEFp SFL1	this work
AGA136	leu2, ste4::KANMX, pRS AgCTS2p lacZ	this work
AGA137	leu2, gpa1::KANMX, pRS AgCTS2p lacZ	this work
AGA138	leu2, pRS 418	this work
AGA139	leu2, sfl1::KANMX, pRS 418	this work
AGA140	leu2, flo8::KANMX, pRS 418	this work
AGA141	leu2, pRS ScTEFp ScSFL1	this work
AGA144	leu2, flo11::KANMX, pRS AgFIG2p lacZ NAT5	this work

Table 2. Shown are strains of *A. gossypii* that were generated and used in this work.

Generated and used Eremothecium gossypii strains

strain	genotype	source
Eremothecium gossy	wt	lab collection
AGA01	flo8::GEN3	this work
AGA02	flo11::GEN3	this work
AGA03	flo11::GEN3	this work
AGA04	flo11::GEN3	this work
AGA05	sfl1::GEN3	this work
AGA06	sfl1::GEN3	this work
AGA07	sfl1::GEN3	this work

Table 3. Shown are strains of *E. gossypii* that were generated and used in this work.

3.2.3. Micromanipulation of *Ashbya* spores

Primary transformants were tested for marker integration via PCR. Primary transformants are heterokaryons, since only some nuclei integrated the resistance marker. They were inoculated in CSM selective media, and the spores were isolated. Spores were incubated in selective AFM at 30°C, 180 rpm for 16 h. Germlings were plated on a part of a selective AFM plate, and single germlings were picked with a Patchman NP2 micromanipulator (Eppendorf) onto another part of the same plate under microscopic observation. Since spores are mononuclear, the growing mycelia should be homokaryons. The absence of the gene of interest was tested via verification PCR.

3.2.4. *Ashbya* DNA-Preparation of transformants

Ashbya mycelium was grown in a baffled flask in AFM at 30°C for 16 h while shaking at 180 rpm. The culture was harvested by vacuum filtration, and a part of it was transferred into a vial containing 500 µl STE buffer and additional 50 µl SDS. After incubation at 65°C for 30 min 200 µl buffer 3 was added, and the vial was incubated on ice for 30 min. This was followed by centrifugation at 13000 rpm for 15 min, and the supernatant was transferred into a new vial containing 750 µl isopropanol. After centrifugation at 13000 rpm for 25 min and washing with 70% ethanol, the DNA pellet was dried and resuspended in 100 µl water.

STE buffer contains 1 M sorbitol (Sigma), 50 mM TRIS-HCl and 100 mM EDTA.
Buffer 3 contains 300 ml 5 M potassium acetate (Merck), 57.5 ml glacial acetic acid, and 142.5 ml of water was added to a final volume of 500 ml.

3.2.5.*Ashbya*DNA-Preparation in big amount

*Ashbya*mycelium was inoculated in 50 ml AFM in a baffled flask and incubated at 30°C and 180 rpm for 2 days. The mycelium was harvested by vacuum filtration and washed once with water before transferred into a 50 ml Falcon tube. Resuspension occurred in 10 ml STE buffer. 10 mg zymolyase were added, and protoplasts were obtained during the following incubation at 37°C with mild shaking at 60 rpm. After adding of 1 ml SDS, the suspension was incubated at room temperature with occasional shaking. The addition of 3.6 ml 3 M calciumacetate was followed by inversion and incubation on ice for 15 min. The tube was further centrifuged at 4000 rpm for 20 min, and the supernatant was combined with 1 vol isopropanol and incubated on ice for 15 min. After the following centrifugation at 4000 rpm for 25 min, the supernatant was discarded and 1 ml 70% ethanol was added. The solution was centrifuged at 4000 rpm for 5 min. After drying the DNA was dissolved in 1 ml water and stored at –20°C.

3.2.6. Purification of*Ashbya*DNA

24 µg RNase A (Sigma) was added to 1 ml DNA suspension and incubated at 37°C for 1 h. 1 vol phenol (Sigma) was added, and the solution was mixed and centrifuged at 13000 rpm for 10 min. The upper phase was transferred into a new vial containing 1 vol chloroform (LAB-Scan analytical sciences, Dublin) followed by centrifugation at 13000 rpm for 10 min. The upper phase was mixed with 1/10 vol 3 M sodiumacetate pH 5.2 and 1 vol isopropanol and centrifuged at 13000 rpm for 25 min. The supernatant was discarded, and the pellet was washed with 70% ethanol followed by drying and dissolving in 150 µl water.

3.2.7.*Ashbya*β-galactosidase assay

The method was modified after Rose, M. *et al.* (1981).
Baffled flasks containing 20 ml AFM and clonNAT were inoculated with 500 µl of a culture that was grown for 16 h at 30°C while shaking at 180 rpm. After 24, 48 or 72 hours of growth under similar conditions, the whole mycelium was harvested by vacuum filtration. It was transferred in 15 ml Falcon-tubes, dissolved in 1-1.5 ml cell disruption buffer to obtain a viscous suspension. After adding 0.5 g glass beads (0.25 – 0.5 mm, Roth), it was frozen in liquid nitrogen. The frozen mixture was shaken on a

vortex shaker until it became liquid in order to disrupt the mycelium. After transferring the compound to a 1.5 ml vial and centrifugation at 13000 rpm for 1 min, the clear supernatant was transferred into a new vial and used for protein measurement according to Bradford and β-galactosidase assay.

For growth assays on plates, 1 ml of a culture that was grown for 16 h was spread on a selective AFM plate and incubated at 30°C. After 24, 48 or 72 hours of growth, respectively, the mycelium of the whole plate was scraped of using a spatula and transferred into a 15 ml Falcon-tube and proceeded the same way as mycelium from a liquid culture.

100 µl of cell extract were put in two 1.5 ml vials each, mixed with 150 µl ONPG solution and incubated at 37°C for 30 min. The control contained 150 µl Z-buffer instead of ONPG solution. After the reaction was stopped by adding 400 µl 1 M Na_2CO_3 (Merck), colour formation was photometrically measured at 420 nm in water dilutions using a BioMate 3 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA).

The β-galactosidase reaction was calculated using the formula

A [nmol/min*mg] = (OD_{420} nm * 1.4)/(0.0045 * protein concentration [mg/ml] * extract volume [ml] * reaction time [min])

The factor 1.4 is a corrector for the reaction volume.

The factor 0.0045 equates to the optical density of 1 nmol/ml o-nitrophenol.

*Ashbya*cell disruption buffer contains 100 mM TRIS, 20% glycerol, 1 mM DTT and 1 mM PMSF (Roth).

Z-buffer contains 60 mM $Na_2HPO_4x2H_2O$ (Merck), 40 mM $NaH_2PO_4x2H_2O$ (Merck), 10 mM KCl (Fluka, Sigma-Aldrich, St. Louis, MO, USA) and 1 mM $MgSO_4x7H_2O$ (Merck).

ONPG solution contains 4 mg/ml ONPG (Fluka) in Z-buffer.

3.2.8. Determination of protein concentrations

The method is based on Bradford, M. (1976).

A calibration curve was generated, using bovine serum albumin dilutions from the Quick Start™ Bovine Serum Albumin Standard Set (BIO-RAD). For protein measurements, the cell extract was diluted using 50 mM KPO_4 pH 7.4. 20 µl extract were mixed with 1 ml Quick Start™ Bradford Dye Reagent (BIO-RAD) and

photometrically analysed at 595 nm using a BioMate 3 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.). The control contained only 50 mM KPO_4.

3.3. Work with yeast

3.3.1. Yeast Transformation with lithium acetate

The method was modified after Gietz, R.D. *et al.* (1995).
50 ml YPD were inoculated with 10-500 µl cell suspension and incubated at 30°C for 16 h while shaking at 180 rpm. The culture was diluted to OD_{600} = 0.3 in 50 ml YPD and grown at 30°C for additional 4 hours while shaking at 180 rpm. OD measurements occurred with a Bio Photometer (Eppendorf). The cell harvest occurred via centrifugation at 4000 rpm for 5 min at room temperature. After washing with 50 ml water, the cells were resuspended in 1 ml water and transferred to a 1.5 ml vial. Using a quick centrifugation at 13000 rpm for 10 s and discarding the supernatant, the yeast cells were resuspended in 1.5 ml lithiumacetate solution.
100 ng of DNA in a maximum volume of 10 µl were put in a 1.5 ml vial together with 100 µg deoxyribonucleic acid from salmon testes (Sigma) and 100 µl of yeast cell suspension. 600 µl of PEG-lithiumacetate solution was added, mixed by vigorous shaking for 15 s and incubated at 30°C for 30 min. Adding of 70 µl DMSO (J.T. Baker, Deventer, Netherlands) to obtain a final concentration of 10% was followed by gentle mixing and incubation at 42°C for 15 min. The transformation mixture was chilled on ice for 1 min, centrifuged at 13000 rpm for 10 s and after removal of the supernatant resuspended in 500 µl selective medium. In order to allow the expression of the resistance marker gene, the cells were incubated at 30°C for 3 h, and 200 µl were plated on selective plates. Transformants occurred after 2 to 4 days at 30°C. The generated and used yeast strains are listed in table 4.

1 l YPD medium consists of 20 g casein peptone (Becton, Dickinson and Company), 10 g yeast extract, 20 g glucose, optional 20 g agar and water up to 1 l. Optional after autoclaving were added geneticin up to a final concentration of 200 µg/ml or clonNAT up to a final concentration of 100 µg/ml.
Lithiumacetate solution consists of 100 mM lithiumacetate (Appli Chem GmbH, Darmstadt, Germany) and 1 x TE.
PEG-lithiumacetate solution contains 40% PEG4000 (Sigma), 100 mM lithiumacetate and 1 x TE and was prepared fresh for each experiment.

Generated and used yeast strains

strain	genotype	source
BY4743	Mat: a/alpha; his3Ä1/his3Ä1; leu2Ä0/leu2Ä0; lys2Ä0/LYS2; MET15/met15Ä0; ura3Ä0/ura3Ä0	EUROSCARF
BY4741	MATa his3Ä1 leu2Ä0 met15Ä0 ura3Ä(	EUROSCARF
YAG001	BY4741, pRS AgFLO5-1p lacZ NAT5	this work
YAG002	BY4741, pRS AgFLO5-2p lacZ NAT5	this work
YAG003	BY4741, pRS AgFLO8p lacZ NAT5	this work
YAG004	BY4741, pRS AgFLO11p lacZ NAT5	this work
YAG005	BY4741, pRS AgSFL1p lacZ NAT5	this work
YAG006	BY4741, pRS AgFIG2p lacZ NAT5	this work
YAG007	BY4743, pRS AgSFL1 GFP	this work

Table 4. Shown are yeast strains that were generated and used in this work.

3.3.2. Yeast DNA preparation

The preparation was done according to Sambrook, J., Russell, D.W. (2001).
5 ml selective medium was inoculated with a colony and incubated at 30°C for 16 h. The cells were collected in a 1.5 ml vial by repeated transferring of culture, centrifugation at 13000 rpm for 20 s and removal of the supernatant. After resuspension in 500 µl STE buffer, 500 µg zymolyase was added followed by incubation at 37°C for 60 min while shaking at 60 rpm. The addition of 50 µl 10% SDS was followed by incubation at 65°C for 30 min. 200 µl buffer 3 was added and after incubation on ice for 30 min, the solution was centrifuged at 13000 rpm for 15 min. The supernatant was transferred into 750 µl isopropanol and centrifuged at 13000 rpm for 25 min. After washing with 70% ethanol, the DNA containing pellet was dried and dissolved in 100 µl water.

STE buffer consists of 1 M sorbitol, 50 mM Tris and 100 mM EDTA.
Buffer 3 contains 300 ml 5 M potassium acetate (Merck), 57.5 ml glacial acetic acid and 142.5 ml of water was added to a final volume of 500 ml.

3.4. Work with *Escherichia coli*

For amplification of plasmids the *E. coli* strain DH5á (F- ö80lacZ ÄM15 Ä(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 ë- thi-1 gyrA96 relA1) was used.

3.4.1. Preparation of*Escherichia coli*DH5α electro competent cells

The method was modified after Dower, W.J. *et al.* (1988).
2 l 2YT medium were inoculated with 40 ml of an *E. coli* DH5α culture that was grown for 16 h at 37°C while shaking at 180 rpm in an Excella E24 Incubator Shaker (New Brunswick Scientific). When an OD_{600} of 0.8 was reached, the culture was chilled on ice for 15 min. The cells were centrifuged at 4000 rpm for 10 min in 300 ml centrifugation bottles, and the supernatant was removed. After resuspending in 200 ml cold water, the centrifugation was repeated and followed by a new resuspension in 100 ml cold water and centrifugation at the same conditions. The cell pellet was resuspended in 20 ml cold 10% glycerol, centrifugated and after discarding of the supernatant resuspended in 1/1 v/v 10% glycerol. This was followed by transfer of the culture to 1.5 ml vials that were stored at –80°C.

1 l 2YT medium contains 16 g tryptic peptone, 10 g yeast extract, 5 g NaCl, optional 20 g agar and water up to 1 l. Optional after autoclaving were added ampicillin (Genaxxon) up to a final concentration of 100 µg/ml or kanamycin (Roth) up to a final concentration of 50 µg/ml.

3.4.2. Transformation of*Escherichia coli*by electroporation

The method was modified after Dower, W.J. *et al.* (1988).
5 µl of plasmid-DNA was placed in a 1.5 ml vial followed by addition of 50 µl of electro competent cells. The mixture was transferred in prechilled 2 mm cuvettes (peqlab) followed by electroporation at 2.5 kV, 25 µF, 200 Ω. 1 ml 2YT was added and mixed with the transformation mixture, followed by transferring into 1.5 ml vials. After incubation at 37°C for 30 min, 150 µl cells were plated on selective 2YT plates and incubated at 37°C for 18 h.

3.4.3.*Escherichia coli*plasmid DNA preparation (miniprep)

The method was modified after Ish-Horowicz, D. and Burke, J.F. (1981).
3 ml of 2YT were inoculated with a single colony and incubated at 37°C 500 rpm for 16 h. 1.5 ml of the culture was transferred in a 1.5 ml vial and centrifuged at 13000 rpm for 2 min in a 260D brushless microcentrifuge (Denville Scientific Inc., NJ, USA). The

removal of the supernatant was followed by resuspension of the cells in 250 µl buffer 1, adding of 250 µl buffer 2 and incubation at room temperature for 5 min with gentle mixing. 250 µl buffer 3 were added, and the solution was centrifuged at 13000 rpm for 15 min. The supernatant was added to 750 µl isopropanol, and the mixture was centrifuged at 13000 rpm for 25 min. Centrifugation at 13000 rpm for 5 min followed the washing of the pellet with 250 µl 70% ethanol. The DNA pellet was dried and resuspended in 50 µl water.

Buffer 1 contains 50 mM glucose, 25 mM Tris, pH 7.5, 10 mM EDTA, pH 8.0 and 100 µg/ml RNase A.
Buffer 2 consists of 200 mM NaOH (Merck) and 1% SDS (Merck).
Buffer 3 contains 300 ml 5 M potassium acetate (Merck), 57.5 ml glacial acetic acid and 142.5 ml of water was added to a final volume of 500 ml.

3.4.4.*Escherichia coli*plasmid DNA preparation (midiprep)

For the *E. coli* plasmid DNA preparation the PureYield^{TM} Plasmid Midiprep System (Promega) was used. 50 ml of 2YT were inoculated with 10 µl of *E. coli* culture and incubated at 37°C, 180 rpm for 16 h. The plasmid preparation was done according to the instruction of the manufacturer. The DNA concentration was measured using Nanodrop model ND-1000 (Thermo Scientific, Wilmington, DE, USA). The plasmids generated and used in this work are listed in table 5. Plasmid maps were generated using Lasergene software version 7 (DNASTAR).

Generated and used plasmids

number	name	source
499	pFA GEN3	Schade
486	pFA AgNAT1	Schade
C136	pFA NAT5	Walther
840	pFA NATMX4	Dünkler
121	pFA KANMX6	Philippsen
651	pRS AgTEFp-lacZ	Dünkler
656	pRS417-AgCTS2p-lacZ	Dünkler
C136	pFA-NAT5	Walther
48	pGUG	Knechtle
120	pSK+	Philippsen
C177	pRS417 (GEN3)	Jorde
186	pRS415	Sikorski, Hieter
C218	pRS418 (NAT5)	Jorde
322	pFA GEN3 AgTEFpmt1	Thorsen
C94	pRS AgRIB3p-lacZ NAT5	Walther
C128	pGEM-ScSFL1-PstI/XbaI	Bauer
C169	pRS417-ScTEF1p-lacZ	Walther

C239	pRS ScTEF1p ScSFL1	this work
C320	pGEM-AgSFL1-ORF+Prom	this work
C321	pRS-AgTEFp-AgSFL1 SalI-SacI	this work
C327	pRS415-AgSFL1-ORF+Prom PstI-SacI	this work
C335	pRS-AgFLO5_1p-lacZ	this work
C336	pRS-AgFLO5_2p-lacZ	this work
C337	pRS-AgFLO8p-lacZ	this work
C338	pRS-AgFLO11p-lacZ	this work
C339	pRS-AgSFL1p-lacZ	this work
C340	pRS-AgFIG2p-lacZ	this work
C401	pRS418-Ag-CTS2p-lacZ-NAT5	this work
C417	pSK+Agflo5-1::kanMX #1	this work
C418	pSK+Agflo5-2::kanMX #1	this work
C419	pSK+Agflo5-1-2::kanMX #1	this work
C420	pSK+Agsfl1::kanMX #2	this work
C421	pSK+Agflo11::kanMX #7	this work
C428	pSK+Agflo8::kanMX #4	this work
C430	pSK+Agfig2::kanMX #6	this work
C446	pRS-AgFLO5-1p-lacZ	this work
C447	pRS-AgFLO5-2p-lacZ	this work
C448	pRS-AgFLO8p-lacZ	this work
C449	pRS-AgFLO11p-lacZ	this work
C450	pRS-AgSFL1p-lacZ	this work
C451	pRS-AgFIG2p-lacZ	this work
C455	pRS-AgTEFp-lacZ, NAT5	this work
C460	pRS415-AgSFL1-GFP	this work
C480	pFA-GEN3-AgSFL1 5'flank #2	this work
C481	pFA-GEN3-AgSFL1 5'flank #3	this work
C482	pSK-AgFLO8 3'flank #1	this work
C483	pSK-AgFLO8 3'flank #4	this work
C487	pRS415-AgFLO8 3'flank #1-1	this work
C488	pRS415-AgFLO8 3'flank #1-4	this work
C506	pFA-GEN3-AgTEFp mt1 w/o 2nd mcs #1	this work
C507	pFA-GEN3-AgTEFp mt1 w/o 2nd mcs #2	this work
C518	pFA-GEN3-AgTEFp SFL1 5'flank	this work
C523	pGEM-AgSTE12	this work
C524	pGEM-AgSTE12	this work
C529	pSK-Agste12::NAT4	this work
C533	pGEM-AgSTE12	this work
C543	pDrive-AgSTE12 rev	this work

Table 5. Shown are plasmids that were generated and used in this work.

3.4.5. Storage of strains

250 µl of culture were mixed with 250 µl medium and 500 µl 50% glycerol (J.T. Baker) in a cryo tube. After 30 min incubation at room temperature the vial was stored at –80°C.

3.5. Microscopy

3.5.1. DAPI staining for nuclear and mitochondrial DNA

200 µl yeast cell suspension or medium containing *Ashbya* mycelium was centrifuged at 13000 rpm for 20 s, and the pellet was resuspended in 100 µl 70% ethanol. After incubation for 5 min and washing with water for two times, the cells were suspended in 50 µl water. 1 µg DAPI (Molecular probes, Invitrogen, Carlsbad, CA, USA) was added, following by incubation at room temperature for 5 min and observation of cells by microscopy.

3.5.2. Microscopical analysis of *Ashbya* and *Saccharomyces*

Microscopical analysis of *Ashbya* and *Saccharomyces* was done with Zeiss Axioplan2 Imaging- or Axio-Imager-Microscope (Zeiss, Jena and Göttingen, Germany) and a MicroMax 1024 CCD camera (Princeton Instruments, Trenton, USA). MetaMorph software tools version 6 (Molecular Devices, Downington, USA) were used.
For visualization of the DAPI signal a filter with 348 nm and of the GFP signal a filter with 489 nm was used.

4. Results

4.1. Molecular analysis of *Ashbya gossypii* flocculation genes and regulators, and comparison to other organisms

Information about genes and proteins were taken from the *Saccharomyces* genome database (www.yeastgenome.org), *Ashbya* genome database (http://agd.vital-it.ch/index.html) and *Candida* genome database (www.candidagenome.org) during the time this work was performed. ClustalW protein alignments were made with Lasergene software MegAlign. The program for domain finding was http://smart.embl-heidelberg.de (Fig.6-9, 11-13).

4.1.1. *FLO5* genes in *A. gossypii*, *S. cerevisiae* and *C. albicans*

S. cerevisiae has one *FLO5* gene with the systematic name YHR211W localised on chromosome VIII with an open reading frame (ORF) of 3227 base pairs (bp) corresponding to a protein length of 1075 amino acids (aa). In *A. gossypii* there are two *FLO5* genes. *FLO5-1* (AFL095W) and *FLO5-2* (AFL092C) are localised inverted on chromosome VI. AgFlo5-1 is with 2535 aa, corresponding to an ORF of 7608 bp, more than twice the size of AgFlo5-2 with 935 aa and an ORF length of 2808 bp. Both proteins share a similarity of 60% and are for AgFlo5-1 32% and for AgFlo5-2 27% identical to ScFlo5, respectively. *C. albicans* has a *FLO5* ortholog with the name *CaYWP1* (orf19.3618) on chromosome 2 with 1602 bp corresponding to 533 aa. The similarity to AgFlo5-1 is 28.7% and to AgFlo5-2 23.6%. CaYwp1 is further 23% similar to ScFlo5.

4.1.2. *FLO11* genes in *A. gossypii*, *S. cerevisiae* and *C. albicans*

ScFLO11 alias *MUC1* or *STA4* has the systematic name YIR019C, is localised on chromosome IX and consists of 4103 bp which equates to 1367 aa. The corresponding gene in *A. gossypii* on chromosome V bears the systematic name AEL023C and has an ORF of 4377 bp, which leads to a protein of 1485 aa. The identity between Flo11 of both organisms is about 28%. *C. albicans* possesses a *FLO11* ortholog as well. *CaEAP1* orf19.1401 is localized on chromosome 2, consists of 1962 bp corresponding to 653 aa and has a similarity of 24.2% to AgFlo11 and 28.7% to ScFlo11.

4.1.3. *FIG2* genes in *A. gossypii*, *S. cerevisiae* and *C. albicans*

ScFIG2 has the systematic name YCR089W. The ORF with 4829 bp is situated on chromosome III and the protein of 1609 aa is 28% identical to the homologue in *A. gossypii* with 561 aa. The corresponding ORF AFR711C on chromosome VI contains 1686 bp. In *C. albicans* a Fig2 ortholog could not be identified.

4.1.4. *FLO8* genes in *A. gossypii*, *S. cerevisiae* and *C. albicans*

ScFLO8 (YER109C) is localised on chromosome V and consists of 2399 bp, which corresponds to 798 aa. The homologue in *A. gossypii* AGL300C is situated on chromosome VII, contains 2820 bp, which leads to a protein of 939 aa, and shows a similarity of only 14% to ScFlo8. The similarity between the *C. albicans* ortholog orf19.1093 with 2379 bp on chromosome 6 and a protein size of 792 aa and AgFlo8 is 24% and between ScFlo8 and CaFlo8 13%. The N-terminal part of Flo8 in all three organisms bears a LisH domain, suggested to be involved in microtubule dynamics (Emes, R.D. and Ponting, C.P. 2001) (Fig. 5 and Fig. 6).

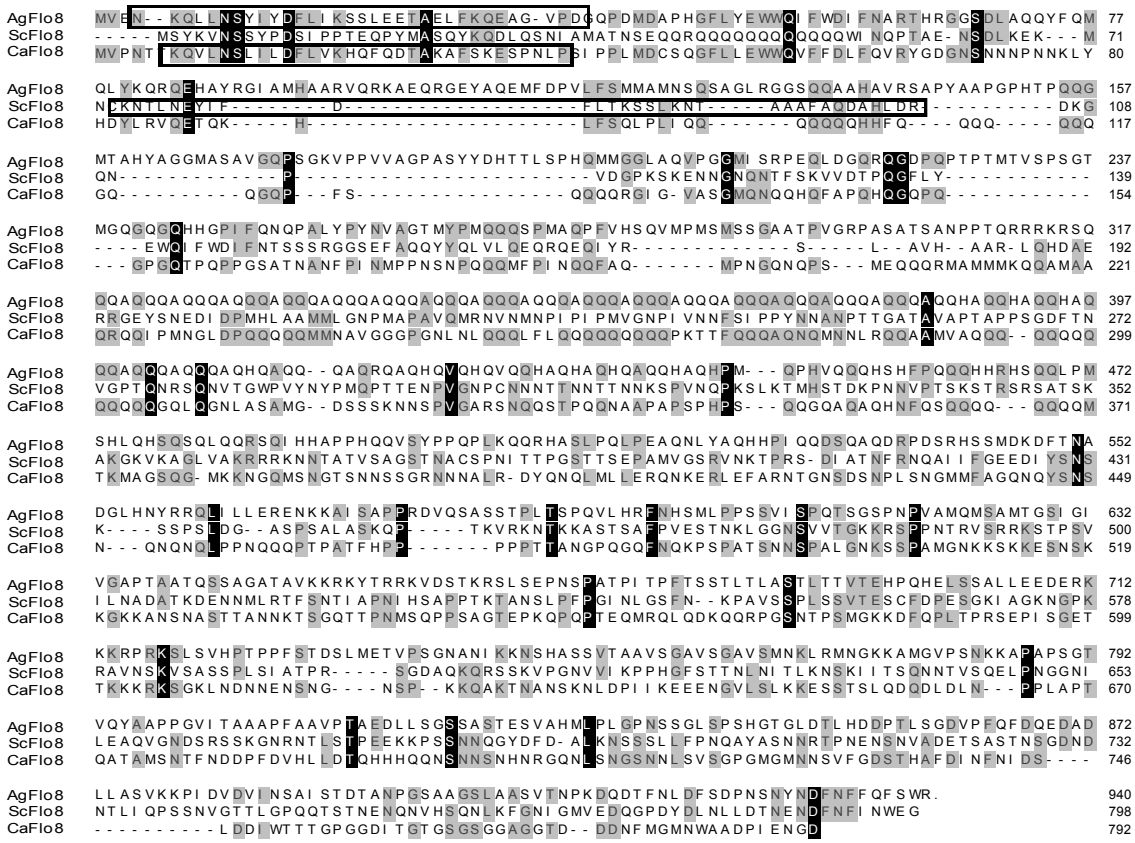


Figure 5. Protein alignment of AgFlo8, ScFlo8 and CaFlo8. Marked with a black frame are the LisH domains.

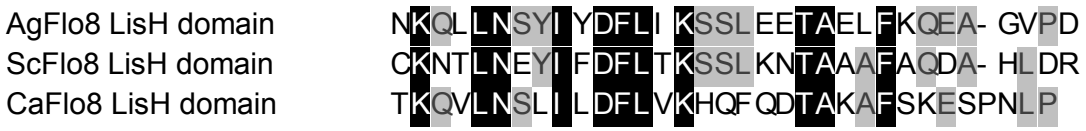


Figure 6. Protein alignment of LisH domains of Flo8 from *A. gossypii, S. cerevisiae* and *C. albicans*. The similarity between the *A. gossypii* and the *S. cerevisiae* domain is about 52%, between AgFlo8 LisH domain and the *C. albicans* counterpart 47% and between ScFlo8 LisH domain and the one in *C. albicans* 41%.

4.1.5. *SFL1* genes in *A. gossypii, S. cerevisiae* and *C. albicans*

The gene *ScSFL1* (YOR140W) has an ORF of 2301 bp localised on chromosome XV. The protein of 766 aa is 25% identical with the homologue of 695 aa in *A. gossypii*. The gene AFR136C with 2088 bp is localised on chromosome VI. ScSfl1 bears a HSTF (heat shock transcription factor) homologous region at its N-terminus, which is conserved in the *A. gossypii* protein. The Myc homologous region of ScSfl1 on the other hand is not detectable in AgSfl1 and CaSfl1 (Fujita, A. *et al.* 1989; this work). The *C. albicans* gene orf19.454 with 2418 bp can be found on chromosome R. The similarity between AgSfl1 and CaSfl1 with 805 aa is about 21% and between ScSfl1 and the *C. albicans* ortholog 18%. The N-terminal part of the protein from all three organisms contains a conserved heat shock factor (Hsf) domain, which marks the proteins as transcription factors. The C-terminus of these domains contains a nuclear localisation sequence that enables the protein to enter the nucleus in order to influence gene expression (Cokol, M. *et al.* 2000) (Fig. 7 and Fig. 8).

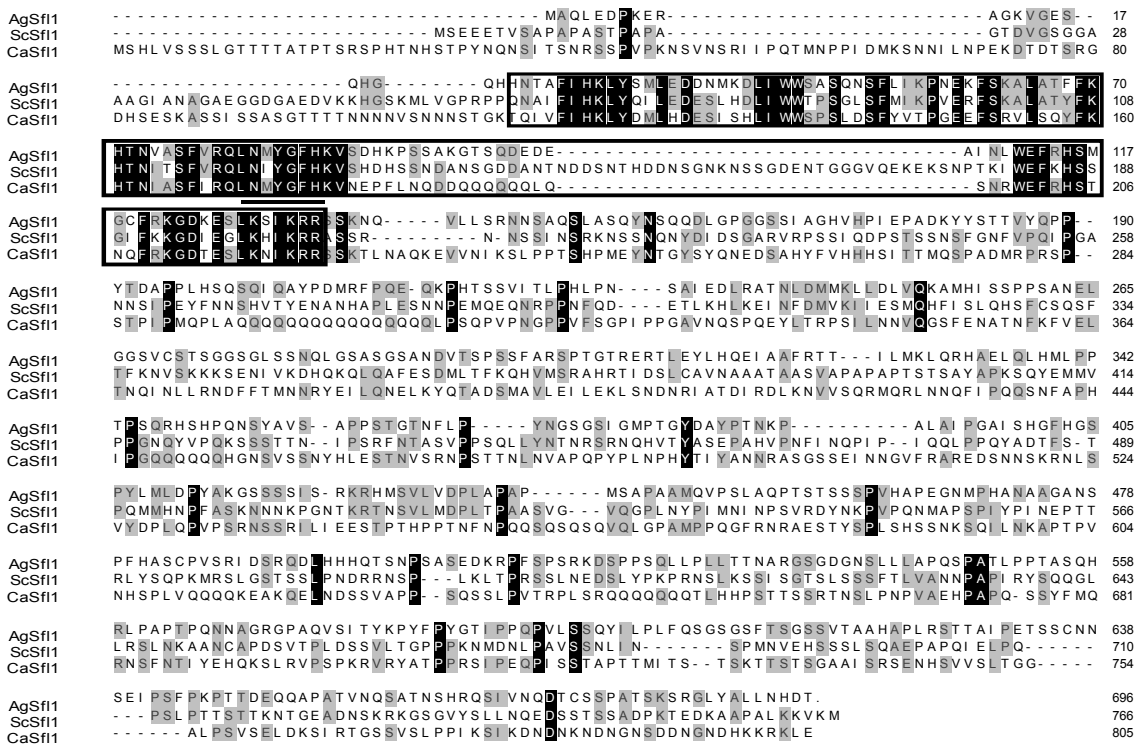


Figure 7. Protein alignment of Sfl1 from *A. gossypii, S. cerevisiae* and *C. albicans* The conserved heat shock factor (Hsf) domain is framed in black, the last 7 amino acids of each Hsf sequence symbolize the nuclear localization sequence (NLS), highlighted with a black line above (http://cubic.bioc.columbia.edu). The NLS is defined as (P/L)KXXKRR.

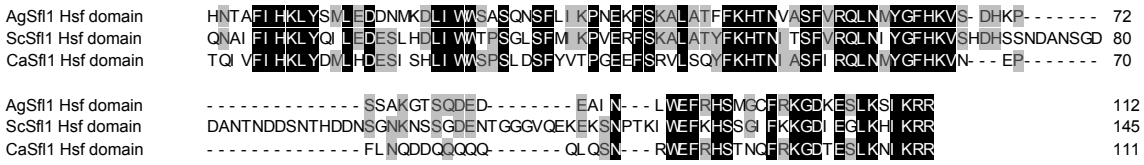


Figure 8. Alignment of the Hsf domain of Sfl1 from *A. gossypii, S. cerevisiae* and *C. albicans*. The domain of *A. gossypii* shows 66% identity to the one from *S. cerevisiae* and 56% identity to the counterpart in *C. albicans* The similarity between ScSfl1 Hsf domain and CaSfl1 Hsf domain is 57%.

4.1.6. *TEC1* genes in *A. gossypii, S. cerevisiae* and *C. albicans*

ScTEC1(YBR083W) has an ORF of 1460 bp on chromosome II leading to a protein of 486 aa. *AgTEC1*(AER177W) on chromosome V contains 2376 bp, and the protein of 791 aa bears 31% identity to the homologue in *S. cerevisiae*.Tec1 from *A. gossypii* is about 11% similar to the *C. albicans* ortholog, and CaTec1 shows a 26% similarity to ScTec1. *CaTEC1*orf19.5908 on chromosome 3 consists of 2232 bp leading to a protein size of 743 aa. The N-terminal part of the proteins contains a TEA domain that enables

the binding of DNA and regulation of gene expression (Buerglin, T.R. 1991) (Fig. 9 and Fig. 10).

Figure 9. Protein alignment of *A. gossypii* Tec1, *S. cerevisiae* Tec1 and *C. albicans* Tec1. Highlighted with a black frame is the TEA domain in all proteins (http://au.expasy.org).

Figure 10. Alignment of the TEA domain of AgTec1, ScTec1 and CaTec1. The domain of *A. gossypii* Tec1 shows 70% similarity to the one in *S. cerevisiae* and 49% similarity to the domain of the *C. albicans* protein. ScTec1 TEA domain and CaTec1 TEA domain share a similarity of 62%.

4.1.7. *STE12* genes in *A. gossypii*, *S. cerevisiae* and *C. albicans*

A protein of 688 aa can be obtained from the 2066 bp ORF of *ScSTE12* (YHR084W) on chromosome VIII. It possesses a 45% identity to AgSte12 with 655 aa, whose 1968 bp ORF (ADR304W) is localised on chromosome IV. The *C. albicans* ortholog CaCph1 shows a 33% similarity to AgSte12 and a 28% similarity to ScSte12. *CaCPH1* orf19.4433 on chromosome 1 consists of 1971 bp leading to a protein size of 656 aa. The STE domain is highly conserved between the three organisms (Yuan, Y.O. *et al.* 1993) (Fig. 11 and Fig. 12).

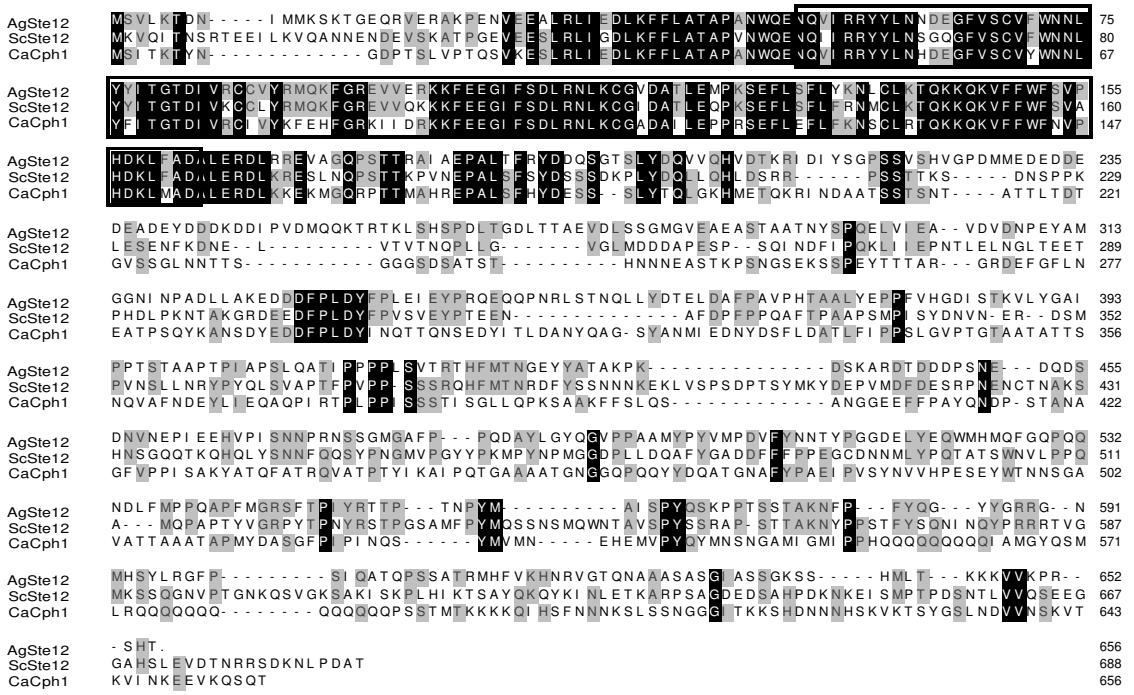


Figure 11. Alignment of AgSte12, ScSte12 and the *C. albicans* homologue CaCph1. Marked with a black frame is the STE domain.

Figure 12. Alignment of STE domains in the Ste12 proteins of *A. gossypii, S. cerevisiae and C. albicans*. The similarity between the AgSte12 STE domain and the counterpart in *S. cerevisiae* is 87%, between AgSte12 STE domain and the one in *C. albicans* 80% and 74% between the *S. cerevisiae* protein domain and the *C. albicans* STE domain.

4.2. Deletion of *Ashbya gossypii* flocculation genes and regulators

Strain *Agleu2* with a deletion of the *LEU2* gene was used for all further deletions if not indicated differently (Mohr, Philippsen). Single deletions of all *A. gossypii* flocculation genes were obtained by using either PCR based gene targeting or cassette-based gene deletion. For PCR based gene targeting plasmids #499 or #486 were used for amplification of the resistance marker gene. In the cassette-based gene deletion approach plasmid #121 was used. Plasmid #499 is a pFA with a kanamycin resistance gene (amino glycoside phosphotransferase from *E. coli*) controlled by *ScTEF2* promoter and terminator referred to as "*GEN3* module". The expression of the gene leads to

resistance against the amino glycoside antibiotic geneticin (G418). Plasmid #486 is a pFA containing a nourseothricin acetyltransferase gene under the control of *AgTEF1* promoter and terminator referred to as "*NAT1* module". The transformants obtained using the nourseothricin resistance gene to disrupt the gene of interest showed a different phenotype under selective and non-selective conditions compared to transformants that were deleted in the same gene using the kanamycin resistance gene. This observation could be due to *AgTEF1* promoter and terminator controlling the nourseothricin acetyltransferase expression. The homology of these elements to the *A. gossypii TEF1* gene could lead to an integration of the disruption cassette not only at the gene of interest but also at the endogenous *TEF1* locus. This second integration could then lead to an additional unfavoured phenotype. Plasmid #121, which was used for cassette-based gene deletions, contains the same resistance gene as #499 controlled by *AgTEF1* promoter and terminator. Since the deletion cassette generated in this approach contains at least 250 bp of the 3' or 5' untranslated region (UTR) of the gene of interest, respectively, a homologous integration into the endogenous *TEF1* locus is unlikely.
Due to these reasons, for phenotypic characterisation only transformants with geneticin resistance, generated by using plasmid #499 or #121, were used.
All camera pictures were taken with a Canon Powershot G5.

4.2.1. Deletion phenotypes of *A. gossypii* adhesins and transcriptional regulators

4.2.1.1. Phenotype on *Ashbya* full medium plates

Deletion mutants of *A. gossypii* flocculation genes and transcriptional regulators were inoculated on full medium plates and incubated at 30°C for 7 days. The colony phenotype of most of the strains was comparable with the precursor strain *Agleu2*. Deletion mutants of the positive and negative regulator of the cAMP-PKA pathway, *FLO8* and *SFL1* respectively, showed a growth delay. The deletion of the transcriptional activator of the MAPK pathway, *TEC1*, resulted in slightly slower growth as well, whereas deletion of the other transcription factor of this pathway, *AgSTE12*, did not result in any phenotype different from the precursor strain (Fig. 13). Attachment of deletion mutants to the plate surface was not impaired either. For this experiment colonies of all strains were inoculated on full medium plates, and after 3 days of growth

at 30°C colonies were removed. The plates were further incubated for one day leading to a similar outgrowth of all strains compared to the wild type (data not shown).

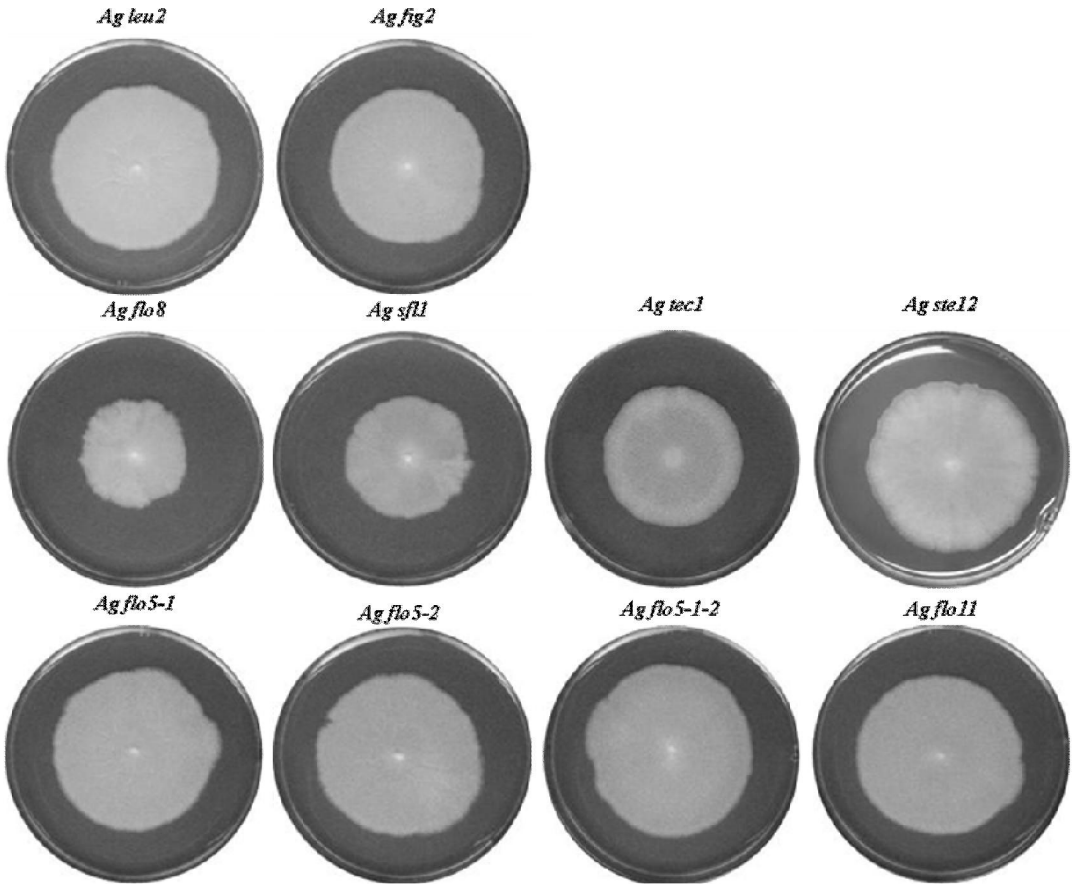


Figure 13. The precursor strain *Agleu2* and deletion mutants of adhesins *FLO5-1, FLO5-2, FLO5-1-2, FLO11, FIG2* and transcriptional regulators of the cAMP-PKA-pathway *FLO8, SFL1* and MAPK-pathway *TEC1, STE12* were inoculated on AFM plates an incubated at 30°C for 7 days. Single deletion of adhesins and the transcriptional regulator *STE12* did not show different colony morphology compared to the precursor strain. Deletion of *AgTEC1* led to a minimal, deletion of *AgFLO8* and *AgSFL1* to a significant growth delay.

4.2.1.2. Phenotype in AFM liquid cultures

The precursor strain *Agleu2* and deletion mutants of *AgFLO5-1, AgFLO5-2, AgFLO5-1-2, AgFLO11, AgFIG2* and of the transcriptional regulators *AgTEC1, AgSTE12, AgFLO8 and AgSFL1* were inoculated in 25 ml AFM and grown at 30°C for 24 h while shaking at 180 rpm. 5 ml were transferred into a glass tube, and pictures were taken. All deletion strains grew similar to the precursor except for *Agflo8, Agsfl1* and *Agtec1* that showed growth delays compared to *Agleu2*. This delay resulted in formation of smaller hyphal balls in liquid culture (Fig. 14). All other deletions behaved like *Agleu2* (data not shown).

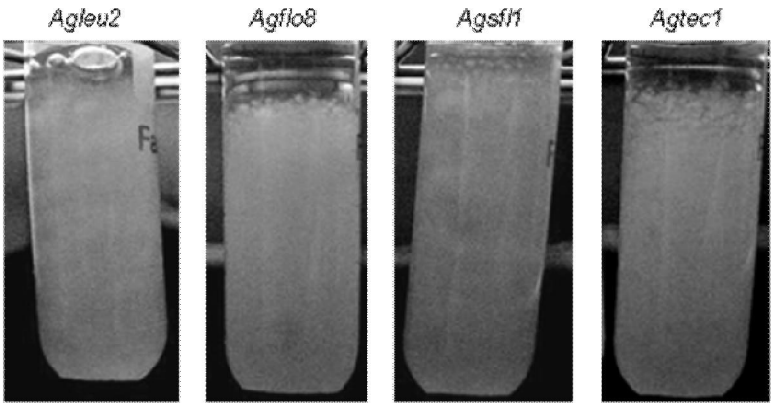


Figure 14. The precursor strain *Agleu2* and the deletion strains *Agflo8*, *Agsfl1* and *Agtec1* were grown in 25 ml AFM at 30°C for 24 h while shaking at 180 rpm. 5 ml were transferred into a glass tube and pictures were taken. Deletion strains *Agflo8*, *Agsfl1* and *Agtec1* formed small mycelia balls indicating slower growth compared to the precursor.

4.2.1.3. Microscopic analysis of *A. gossypii* deletion strains grown on full medium

To analyse growth of the deletion mutants on microscopic level, spore solutions were plated on object slides that were covered with full medium and incubated at 30° for one to two days before examined microscopically (Fig. 15). Young mycelia are characterised by lateral branching whereas mature mycelium shows dichotomous tip growth. The microscopic analysis showed the fast outgrowth of hyphae of *Agleu2* resulting in first lateral then Y-shaped branching of hyphae in growing zones. *Agsfl1* showed the same branching pattern, but slower growth resulted in more compact colonies with shorter outgrowing hyphae. After an additional day of incubation *Agsfl1* hyphae behaved like earlier *Agleu2*, showing typical dichotomous branching. The phenotype observed for *Agsfl1* was similar in *Agflo8*.

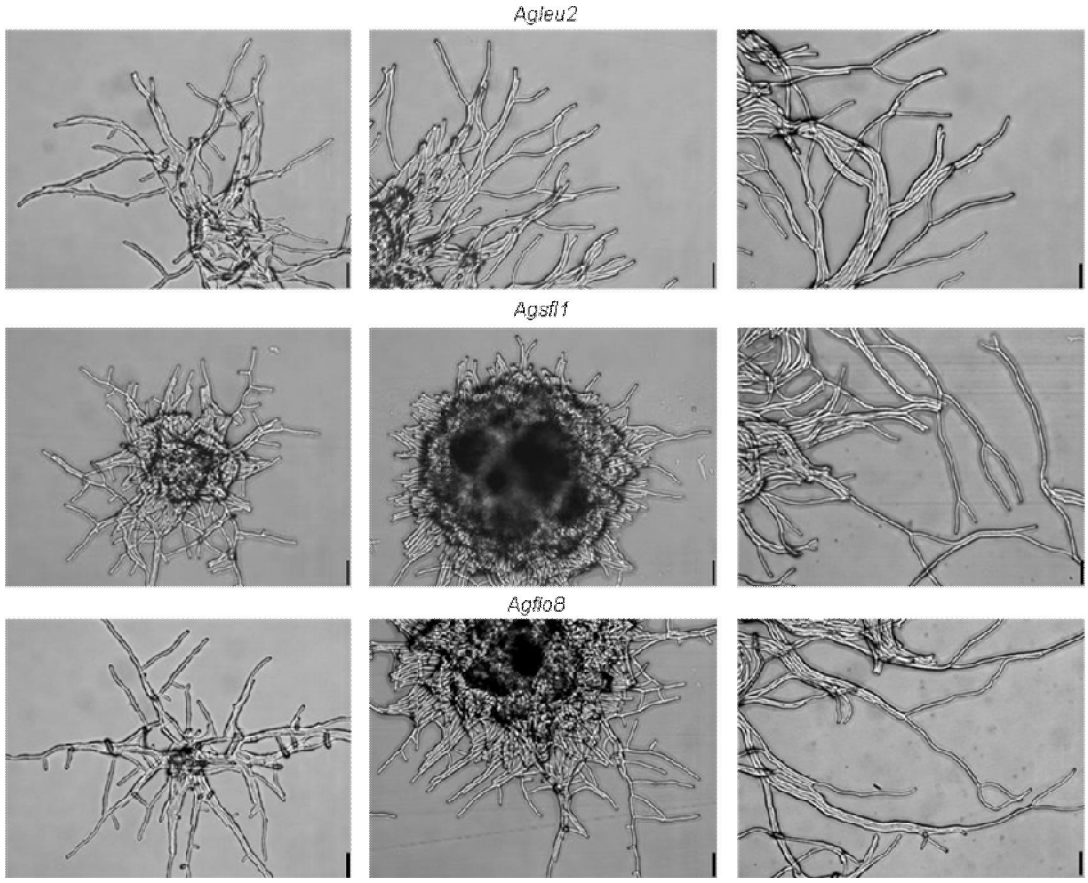


Figure 15. Spores of the deletion mutants *Agflo8* and *Agsfl1* as well as the precursor strain *Agleu2* were inoculated on object slides covered with AFM and incubated at 30°C. Pictures were taken after one day of growth, the bar symbolises 100 µm. The last picture to the right of *Agsfl1* and *Agflo8* shows hyphae at the age of two days. *Agsfl1* and *Agflo8* showed a slower growth compared to the precursor strain resulting in compact hyphal aggregates. However after one additional day the outgrowing hyphae resembled the phenotype of *Agleu2* of the day before.

Mature *A. gossypii* cultures on solid full medium form aerial hyphal aggregates similar to synnemata. These aggregates occur on the whole colony except for the outermost fringe. They seem to colocalise with sides of sporulation but are not necessary for it, because liquid grown mycelium sporulates without forming synnemata, and colonies on minimal medium sporulate with barely developed aerial aggregates. A spore suspension of all deletion strains was inoculated on full medium covered object slides and incubated at 30°C for 3 days before microscopically analysed (Fig. 16). Aerial hyphal aggregates of all deletion mutants showed similar amounts and sizes compared to the precursor strain, but those of *Agflo8* and *Agsfl1* were slightly smaller. This resembles the reduced growth of the mutants, since after more days of growth synnemata reached sizes similar to *Agleu2*

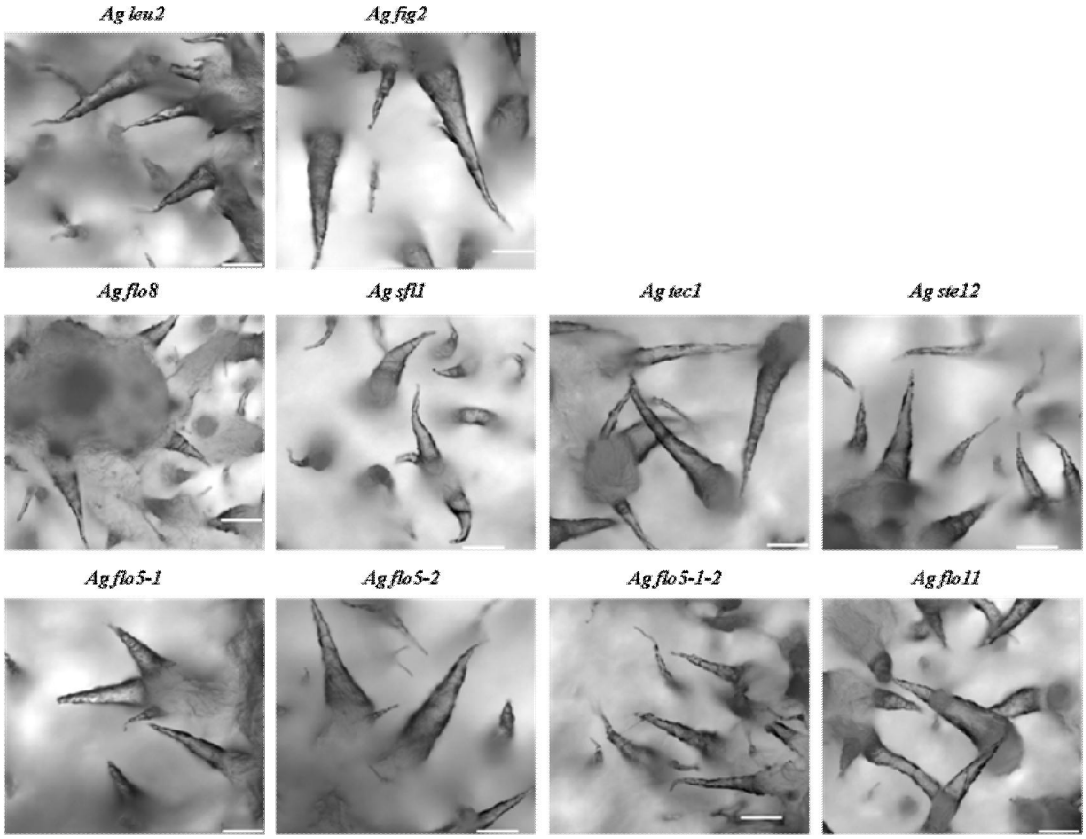


Figure 16. Strains were grown on object slides covered with AFM at 30°C for 3 days before pictures of aerial hyphal aggregations were taken. The bar symbolizes 100 µm. The size of these synnemata was approximately the same in deletion mutants and precursor strain. Their size in *Agflo8* and *Agsfl1* appeared a bit smaller but was the same after some days of further incubation.

4.2.1.4. Phenotype of deletion mutants on CSM plates

Deletion mutants were tested for growth on CSM, which is usually used for induction of sporulation and leads to slower growth of colonies compared to full medium. Inoculants on CSM plates were incubated at 30°C for 10 days before pictures were taken. The growth resembled that on AFM plates with no different phenotype for deletion mutants of adhesin genes (*FLO5-1*, *FLO5-2*, *FLO5-1-2*, *FLO11* and *FIG2*) and of *STE12* compared to the precursor. *Agflo8*, *Agsfl1* and *Agtec1* showed growth delays compared to *Agleu2* (Fig. 17).

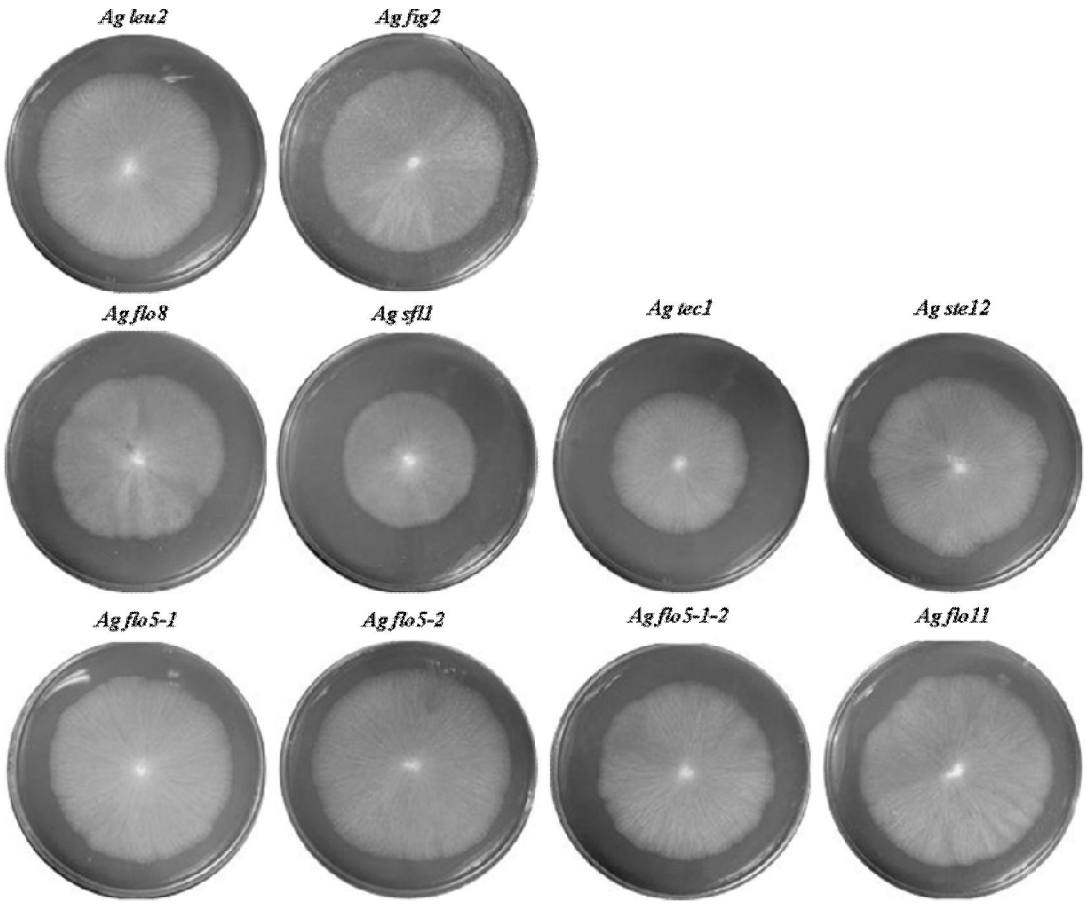


Figure 17. The indicated strains were inoculated on CSM plates an incubated at 30°C for 10 days. Single deletion of adhesins and the transcriptional regulator Ste12 did not show different colony morphology compared to the precursor strain. Deletion of *AgTEC1, AgFLO8* and *AgSFL1* lead to a significant growth delay as was observed on full medium plates.

4.2.1.5. Microscopic analysis of *A. gossypii* deletion strains grown on CSM

Although *A. gossypii* sporulates on CSM medium there are barely aerial hyphal aggregations visible, making it unlikely for them to be involved in spore production. Spore solutions of deletion mutants were inoculated on CSM covered object slides and incubated at 30°C for 4 days before microscopically analysed. Identical synnemata formation could be observed for all deletion mutants except for *Agflo8, Agsfl1* and *Agtec1*, that showed reduced production concerning amount and size compared to *Agleu2* (Fig. 18). This could be due to their growth delay since a few more days of growth lead to increased amounts and sizes of hyphal aggregates in the named strains.

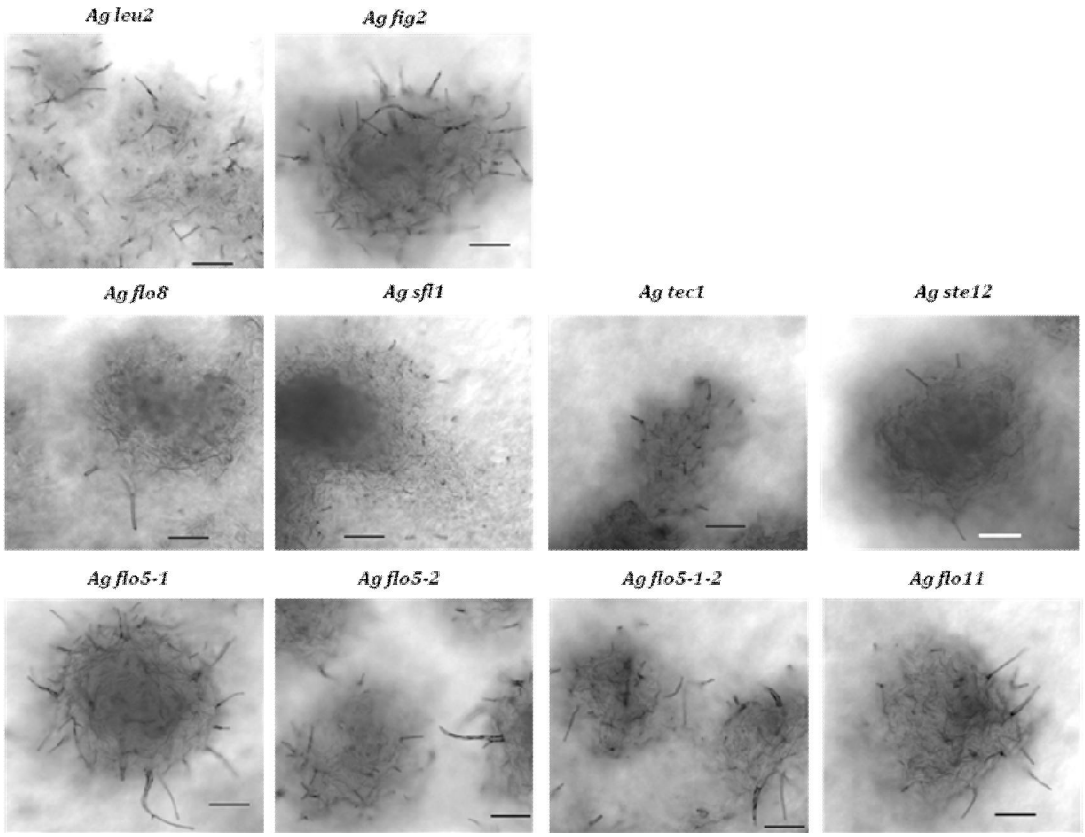


Figure 18. The indicated strains were inoculated on object slides covered with CSM an incubated at 30°C for 4 days. The bar represents 100 µm. Single deletions of adhesins and the transcriptional regulator Ste12 did not show different morphology of aerial hyphal aggregations compared to the precursor strain. Deletion of *AgTEC1*, *AgFLO8*and *AgSFL1* lead to a significant reduction in synnemata number and size.

4.2.1.6. Phenotype of deletion mutants on AFM plates containing 1 M glucose

The growth behaviour of deletion mutants was also tested on AFM containing 1 M glucose, resembling more the natural environment of *Ashbya gossypii*as a plant pathogen on citrus fruits. For this purpose, strains were inoculated on AFM 1 M glucose plates and incubated at 30°C for 7 days before pictures were taken. All strains showed a smaller colony size compared to growth on AFM. This result shows that a high sugar concentration has a growth reducing influence on *A. gossypii*Single or double deletions of *FLO5-1* and *FLO5-2*, deletions of *FLO11*, *FIG2*or *STE12*behaved like the precursor strain. Deletion strains *Agflo8*, *Agsfl1*and to a less extend *Agtec1* showed reduced growth but without the same growth restriction as on AFM, which could be due to insensitivity against high sugar concentrations (Fig. 19).

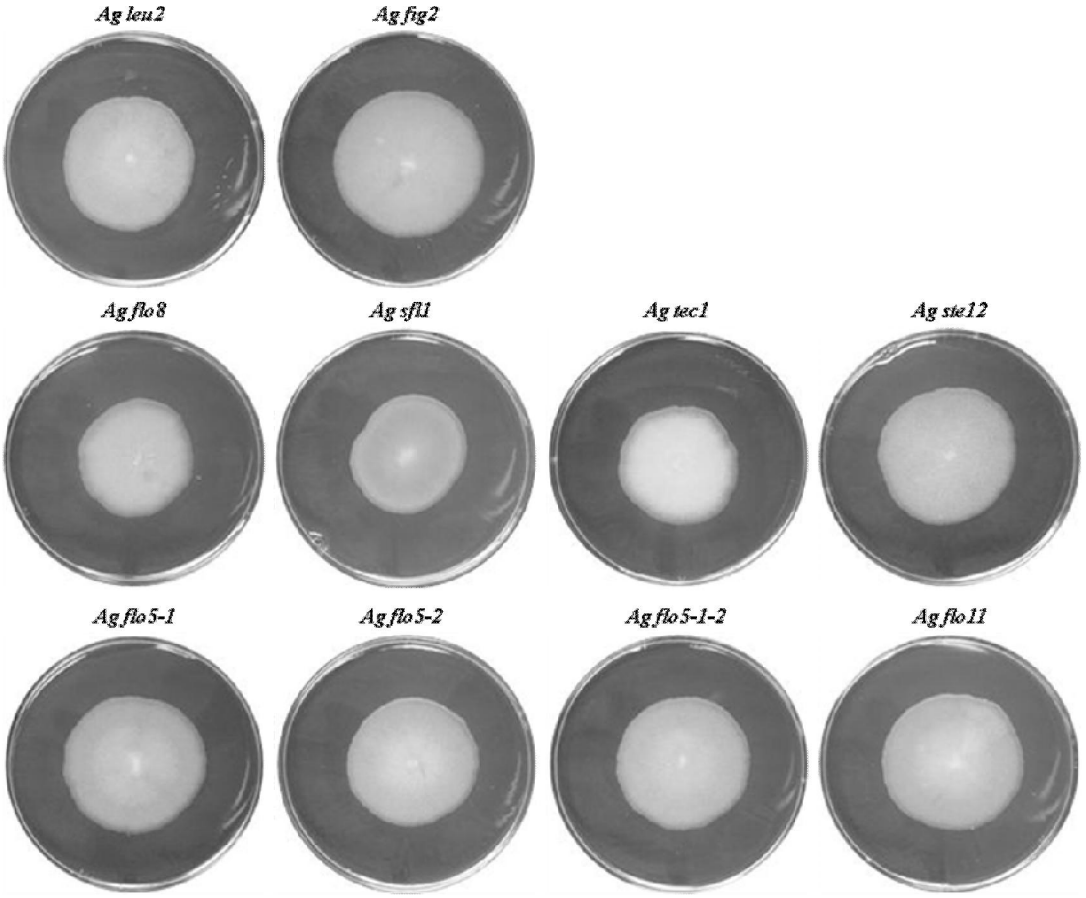


Figure 19. The indicated strains were inoculated on AFM plates with 1 M glucose an incubated at 30°C for 7 days. All strains showed slower growth on high sugar concentrations compared to AFM medium. Single deletion of adhesins and *STE12* did not show different colony morphology compared to the precursor strain. Deletions of *AgTEC1*, *AgFLO8* and *AgSFL1* showed slightly smaller colony sizes than *Agleu2* but not as obvious as on AFM plates.

4.2.1.7. Microscopic analysis of *A. gossypii* deletion strains grown on AFM 1 M glucose

A spore suspension of all deletion strains was inoculated on object slides covered with full medium containing 1 M glucose and incubated at 30°C for 3 days before microscopically analysed (Fig. 20). Aerial hyphal aggregates of all deletion mutants showed similar amounts and sizes compared to the precursor strain. Obvious was the smaller size of synnemata compared to colonies grown on AFM. This resembles the general slowed growth of all strains on high glucose concentrations. Since the difference between precursor strain and *Agflo8*, *Agsfl1* or *Agtec1* is not present any more this could indicate an insensitivity of these mutants against high sugar concentrations.

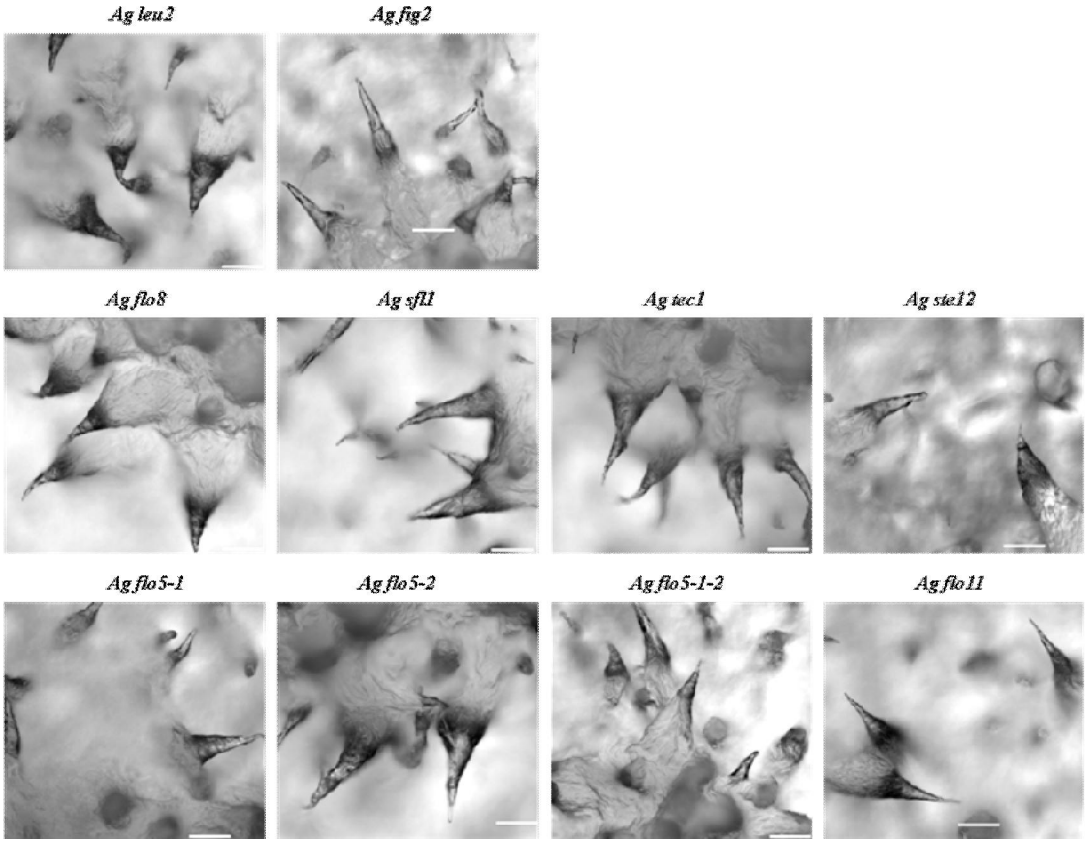


Figure 20. Strains were grown on object slides covered with AFM 1 M glucose at 30°C for 3 days before pictures of aerial hyphal aggregations were taken. The bar symbolizes 100 µm. The size of these synnemata was approximately the same in deletion mutants and precursor strain.

4.2.1.8. Phenotype of deletion mutants on AFM plates containing 1 M fructose

Since the natural habitat of *A. gossypii* are cotton plants or citrus fruits it could as well be fructose playing a major role in the C-metabolism. Growth on AFM containing 1 M fructose was examined. Deletion strains were inoculated on accordant plates and incubated at 30°C for 7 days before pictures were taken. The result resembled that of colonies grown on AFM 1 M glucose. All strains showed a slower growth on the high sugar concentration compared to AFM. Except for a smaller colony size of *Agflo8*, *Agsfl1* and *Agtec1* all other deletion mutants were similar to the precursor strain (Fig. 21). Again the size difference between the three named mutants and *Agleu2* was not as big as on AFM leading to speculation about insensitivity against high sugar concentrations.

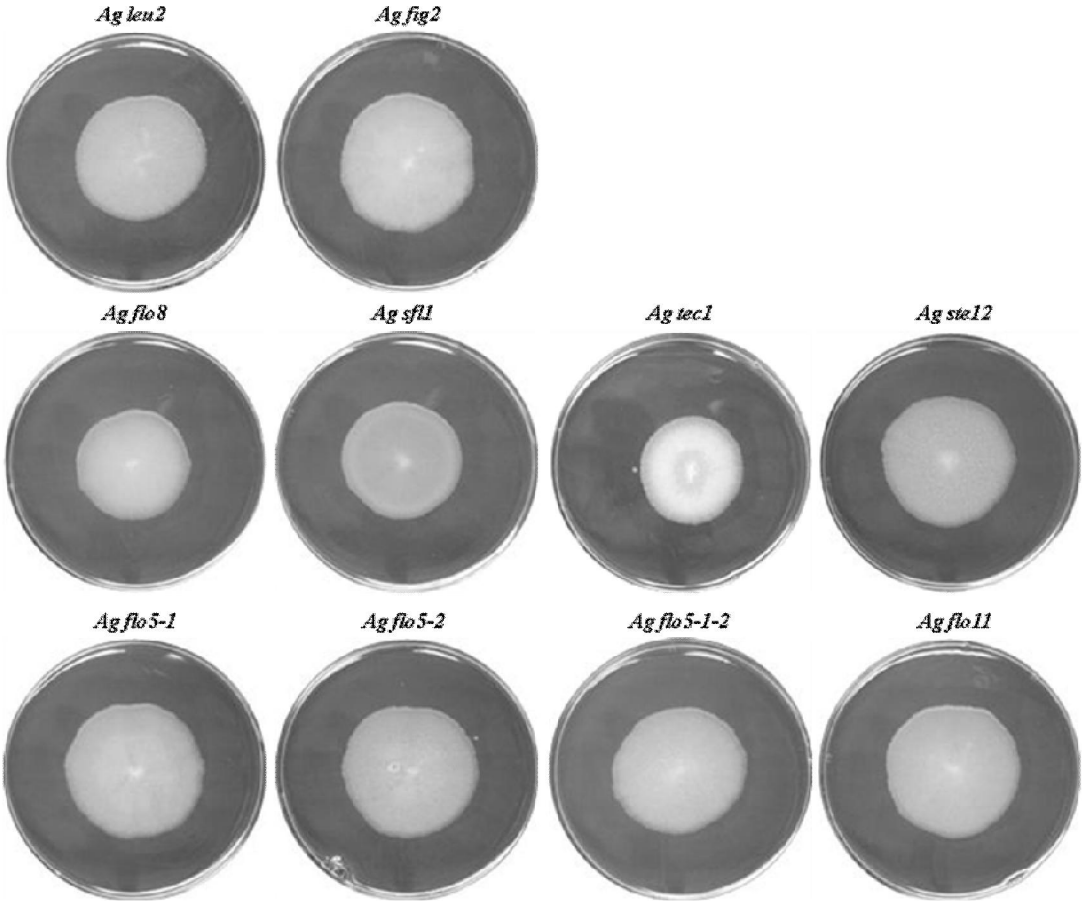


Figure 21. The indicated strains were inoculated on AFM plates with 1 M fructose and incubated at 30°C for 7 days. All strains showed slower growth on high sugar concentrations compared to AFM medium. Single deletion of adhesins *STE12* did not show different colony morphology compared to the precursor strain. Deletions of *AgTEC1*, *AgFLO8* and *AgSFL1* showed slightly smaller colony size than *Agleu2* but not as obvious as on AFM plates.

4.2.1.9. Microscopic analysis of *A. gossypii* deletion strains grown on AFM 1 M fructose

A spore suspension of all deletion strains was inoculated on object slides covered with full medium containing 1 M fructose and incubated at 30°C for 3 days before microscopically analysed (Fig. 22). Aerial hyphal aggregates of all deletion mutants showed similar amounts and sizes compared to the precursor strain. Obvious was the smaller size of synnemata compared to colonies grown on AFM. This resembles the general slowed growth of all strains on high fructose concentrations. Since the difference between precursor strain and *Agflo8*, *Agsfl1* or *Agtec1* was not present any more this could indicate an insensitivity of these mutants against high sugar concentrations.

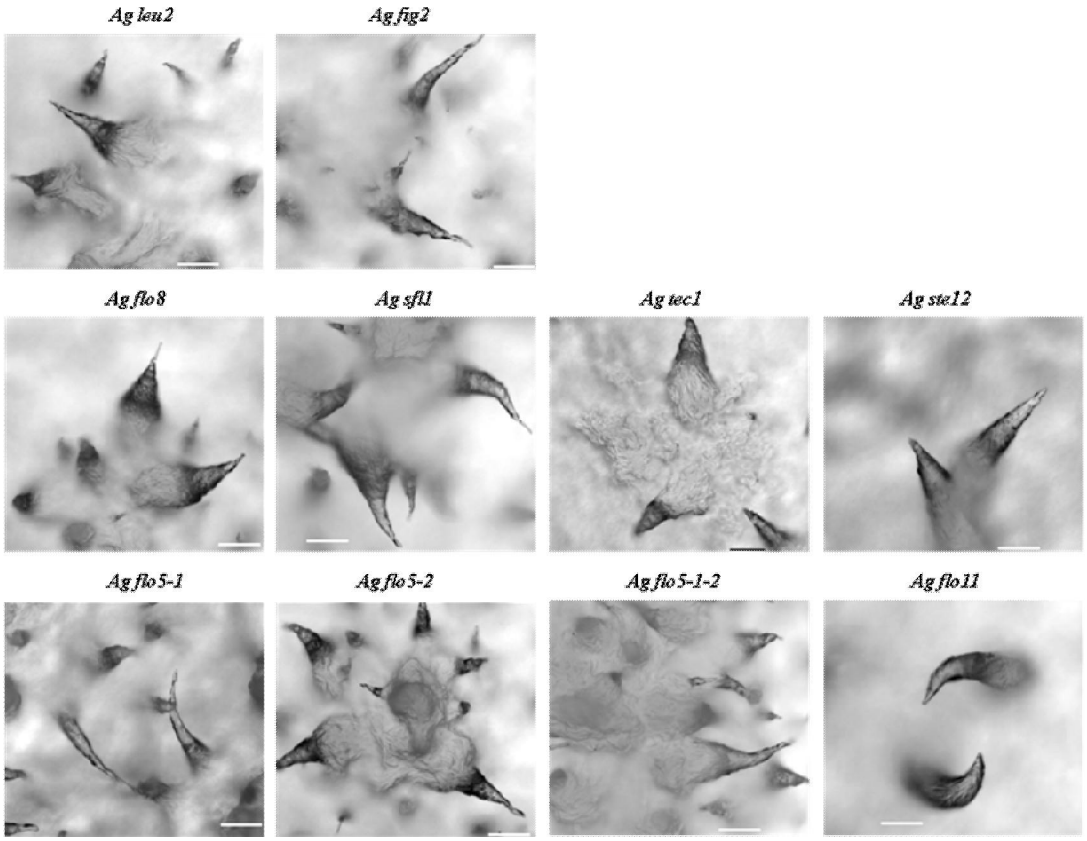


Figure 22. Strains were grown on object slides covered with AFM 1 M fructose at 30°C for 3 days before pictures of aerial hyphal aggregations were taken. The bar symbolizes 100 µm. The size of the synnemata was approximately the same in deletion mutants and precursor strain.

4.2.1.10. Phenotype of deletion mutants on AFM plates containing 70 mM mannose

The flocculins of *S. cerevisiae* are of the Flo1 type and repressible with mannose. According to Bayly, J.C. *et al.* (2005), 70 mM mannose leads to complete loss of flocculation. To test this in *A. gossypii* liquid cultures and plates with AFM containing 70 mM mannose, instead of the usual 100 mM glucose, were inoculated with *Agleu2* and all deletion strains. Liquid cultures were incubated at 30°C for 24 hours while shaking at 180 rpm. Plates were incubated at 30°C for 7 days (Fig. 23). The phenotype of all strains resembled growth on AFM plates. Deletion strains did not show any difference compared to *Agleu2* except *Agtec1*, which had a slight growth delay, and *Agflo8* and *Agsfl1* that grew significantly slower than the precursor strain. Strains grown in liquid cultures behaved like those grown in AFM. Since mannose represses Flo1 type flocculation in *S. cerevisiae* this impact could not be observed in *A. gossypii*. Either flocculins in this organism are repressible with other sugars or a negative influence on

flocculins does not lead to an altered phenotype, implying other proteins being involved in filamentous growth and attachment to surfaces.

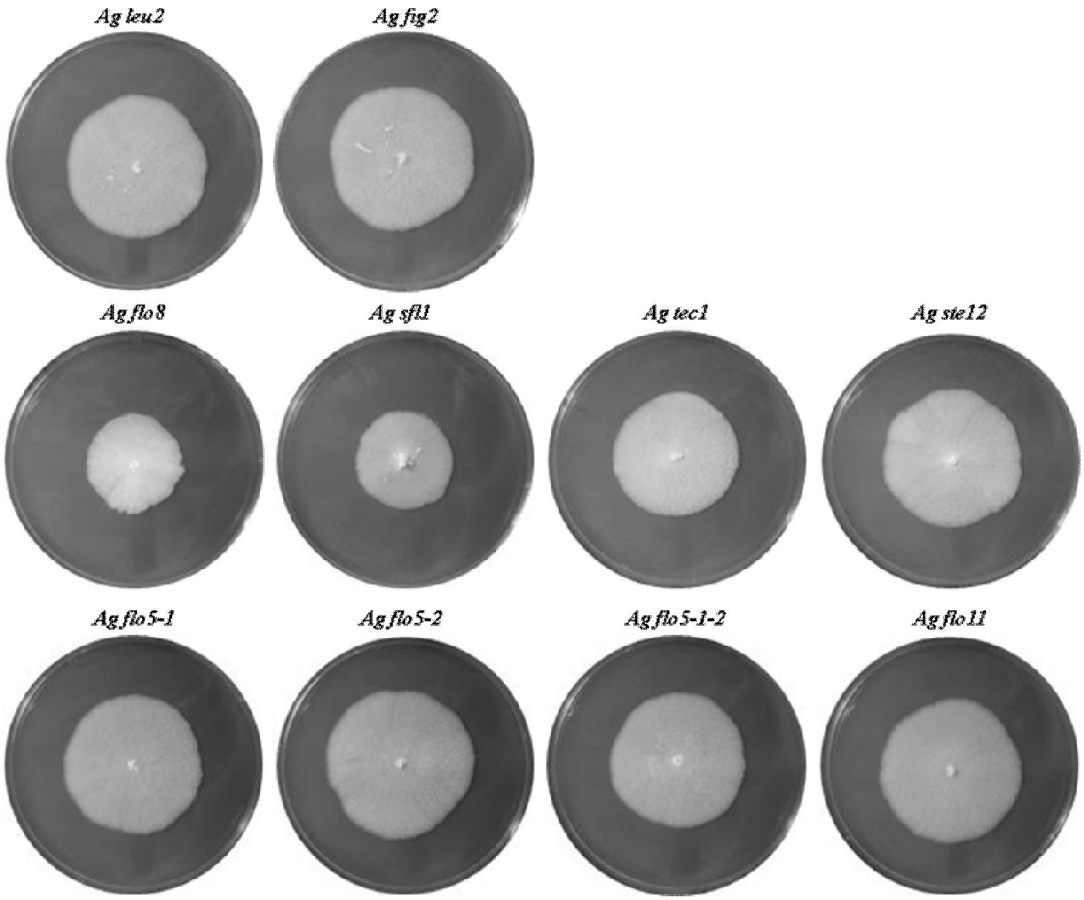


Figure 23. AFM plates containing 70 mM mannose instead of glucose were inoculated with all deletion strains and their precursor *Agleu2*, incubated at 30°C for 7 days and pictures were taken. The growth resembled thus on full medium with a slight growth delay for *Agtec1* and a more severe delay for *Agflo8* and *Agsfl1* compared to the precursor strain.

4.2.2. Growth delay of *AgSFL1* and *AgFLO8* deletion mutants in *Eremothecium gossypii*

The isolate *Eremothecium gossypii* which is characterised by its strong riboflavin production and abundant sporulation, was used for PCR based gene deletion of *SFL1* and *FLO8* as well. The same reduced growth phenotype as in *A. gossypii* could be observed and was quantified by measurement of colony size during growth on full medium plates at 30°C (Fig. 24 and Fig. 25). This result indicates that the growth phenotype is not strain specific but also occurs in other strain backgrounds.

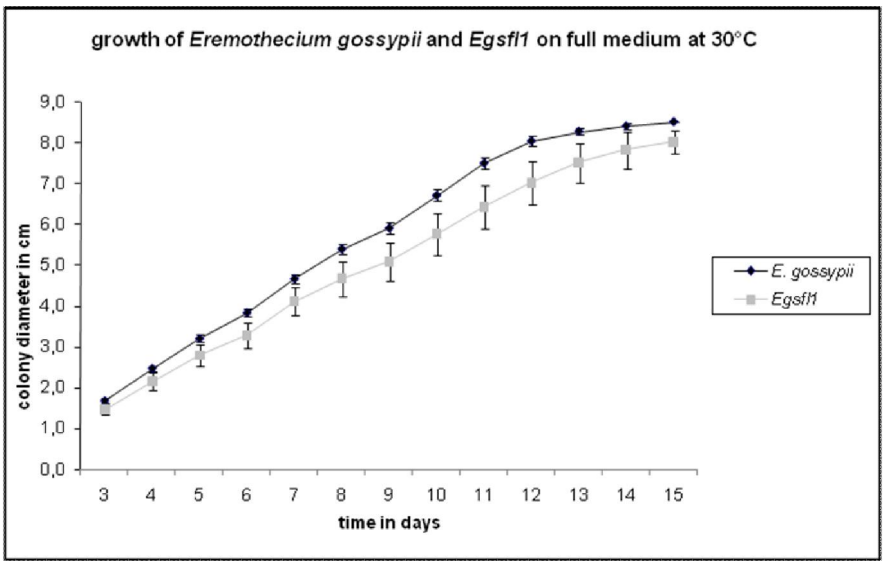


Figure 24. Mycelial inoculates of *Eremothecium gossypii* and *Egsfl1* were grown on AFM plates at 30°C and colony size was measured. The growth delay of *Egsfl1* was significant and the colony sizes of precursor strain and deletion mutant converged when growth ceased.

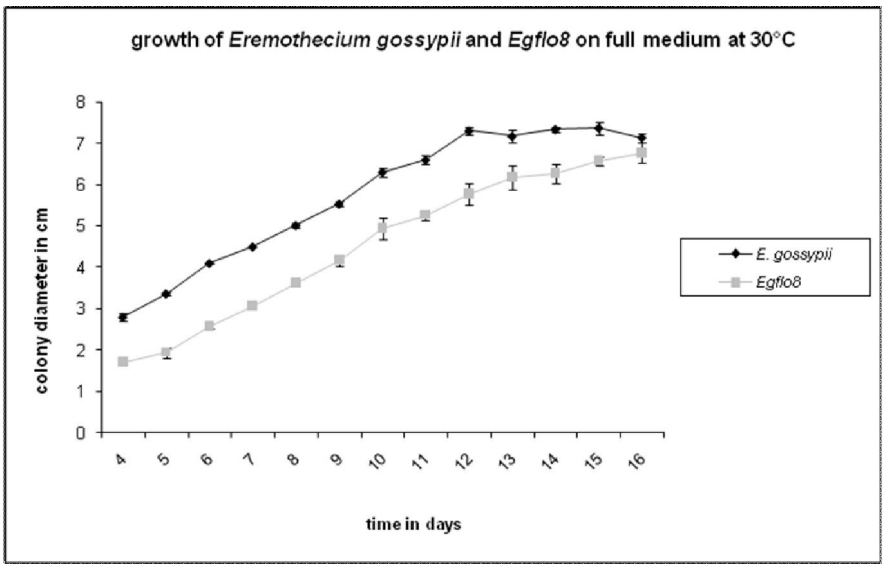


Figure 25. Mycelial inoculates of *Egflo8* and its precursor strain *Eremothecium gossypii* were grown on AFM plates at 30°C and colony size was measured. *Egflo8* showed a significant growth delay but colony sizes converged to the level of the precursor strain when growth ceased.

4.3. Overexpression of *AgSFL1*

4.3.1. Plasmid based overexpression of *AgSFL1*

The hypothesis that deletion of the suppressor of flocculation should lead to a higher expression of flocculation genes and maybe stronger attachment to the agar surface could not be confirmed, and only a growth delay could be observed. An overexpression

of *AgSFL1* on the other hand should lead to reduced expression of flocculation genes and therefore reduced attachment. Since this was not observed with single deletions of flocculation genes, maybe more than one gene suppressed by AgSfl1 is downregulated in an *SFL1* overexpression mutant and leads to the speculated phenotype.

The ORF of *A. gossypii SFL1* was amplified with primers 5'-AgSFL1-SalI starting with the first codon ATG and 3'-AgSFL1-SacI finishing 119 bp after the stop codon TAA. The choice of primers introduced a *SalI* cleavage site at the 5' end and a *SacI* cleavage site at the 3' end of the PCR product, which were used to digest the amplified DNA. The plasmid #651 contains a *Streptococcus thermophilus lacZ* gene under the control of *AgTEF1* promoter and a pFA-GEN3 module. The *lacZ* is flanked by an *XhoI* and a *SacI* cleavage site, which were used in a digest to remove the *lacZ* gene.
The *AgSFL1* ORF was inserted in the plasmid after the *AgTEF1* promoter by ligation. After propagation in *E. coli* DH5a and analytical digest, *Agleu2* was transformed with the resulting plasmid #C321 (Fig. 26).

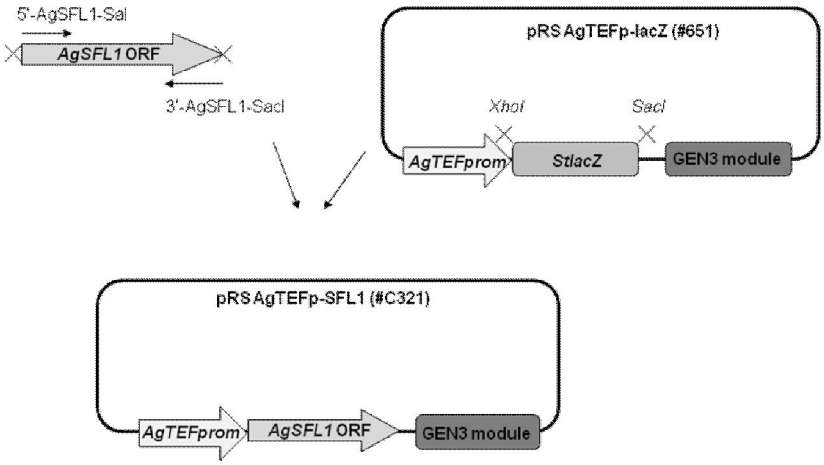


Figure 26. Construction scheme of pRSAgTEFp-SFL1. The ORF of *AgSFL1* was amplified and restriction sites for *SalI* and *SacI* were introduced. PRSAgTEFp-lacZ was used for replacing the *lacZ* ORF with *AgSFL1* ORF leading to its regulation via the *AgTEF1* promoter.

Strains of transformants overexpressing *AgSFL1* were inoculated on AFM G418 and incubated at 30°C for 7 days. As a reference strain *Agleu2* was transformed with pRSAgTEFp-lacZ, to create resistance against geneticin and to avoid effects due to different media if *Agleu2* had been taken as a reference (Fig. 27).

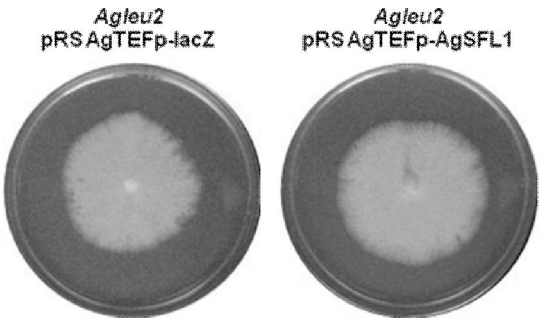


Figure 27. Colony phenotype of *Agleu2*pRSAgTEFp-lacZ and *Agleu2*pRSAgTEFp-AgSFL1 on selective full media (AFM G418) after growth at 30°C for 7 days. To compare growth on selective media, *Agleu2* was transformed with plasmid #651 to generate resistance against geneticin and used as growth control.

There is no colony phenotype visible between an *A. gossypii* strain overexpressing *AgSFL1* and a reference strain. A possible explanation could be the unequal distribution of plasmids in the mycelium. To exclude a plasmid based effect, a strain overexpressing *SFL1* from the natural gene locus was created.

4.3.2. Overexpression of *AgSFL1* from the natural gene locus

Plasmid #C321 was used to amplify the *AgTEF1* promoter together with the first 267 bp of the *AgSFL1* ORF using primer 5'-AgTEFp-KpnI and AgSFL1-rev-EcoRI. The newly introduced restriction sites were used in a digest to generate sticky ends. Plasmid #C506 (pFA GEN3) was digested with the same restriction enzymes, and the PCR fragment was ligated into the vector behind the resistance gene. #C506 was constructed from #499 (pFA GEN3) by digest with *BamH*I-*Bgl*II and religation, which eliminated the second multiple cloning site. Further the fragment together with the *GEN3* module was amplified from the plasmid using primer AgSFL1-rev-EcoRI and S1-AgSFL1, which creates a 45 bp homologous region to the *AgSFL1* 5'UTR. The PCR fragment was used for transformation of *Agleu2* and integrated by homologous recombination at the beginning of the *AgSFL1* ORF (Fig. 28). The resulting transformants were verified by PCR and cultivated for sporulation. After micromanipulation of the germlings, the homokaryotic state was verified by PCR.

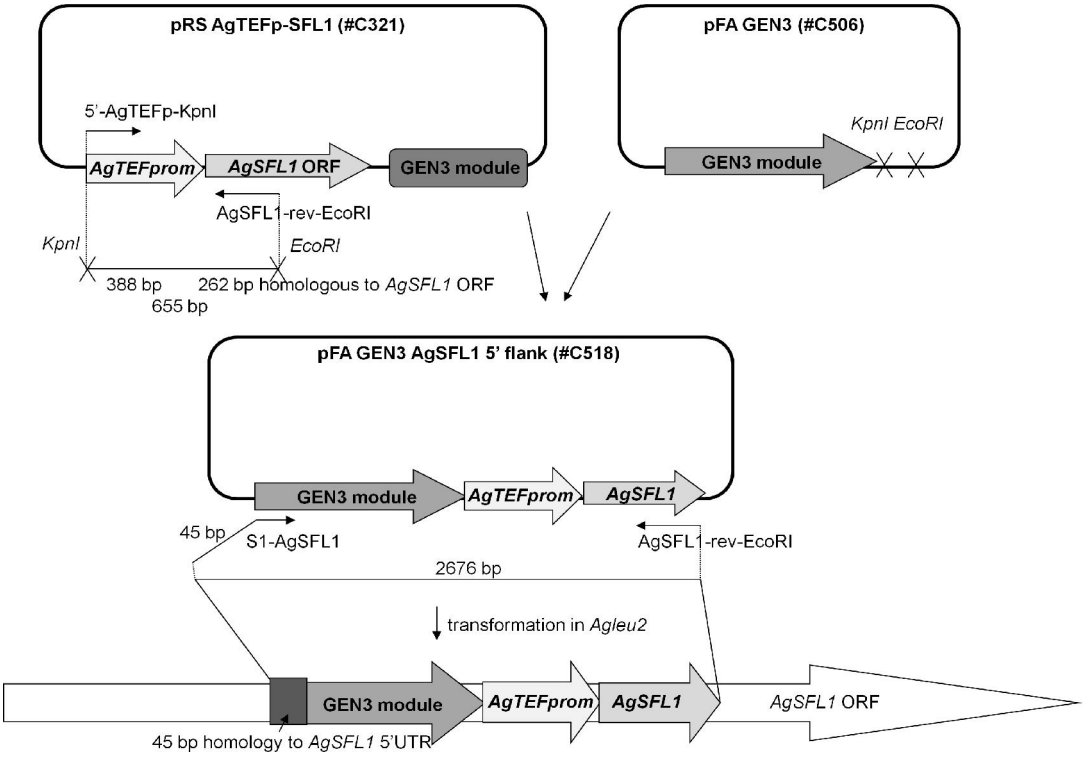


Figure 28. Construction scheme of the *AgSFL1* overexpressing strain. The *AgTEF1p* was PCR amplified together with 262 bp of the 5' region of the *AgSFL1* ORF from pRSAgTEFp-SFL1 introducing restriction sites for *KpnI* and *EcoRI*. The fragment was then placed behind the *GEN3* module in pFAGEN3 using the same restriction sites. Fragment and *GEN3* module were amplified by PCR introducing a 45 bp homologous region to the *AgSFL1* 5' UTR and used for homologous integration in the 5' region of the *AgSFL1* gene.

Colonies of *Agleu2* and the Ag*SFL1* overexpressing strain were inoculated on AFM plates and incubated at 30°C for 7 days before pictures were taken (Fig. 29).

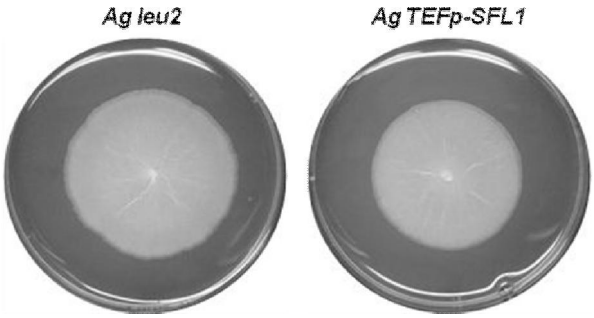


Figure 29. Colony phenotype of *Agleu2* and *AgTEFp-SFL1* on AFM plates after growth at 30°C for 7 days. There was no phenotypic difference between the two strains visible.

As well as the plasmid based overexpression of *AgSFL1* the overexpression from the natural locus did not result in a colony phenotype.

4.3.3. Heterologous overexpression of *ScSFL1* in *A. gossypii*

Overexpression experiments were also done with ScSfl1. For this purpose *ScSFL1* was amplified from plasmid pGEM-ScSFL1 (#C128) using primers 5'ScSFL1-PstI and 3'ScSFL1-XbaI, introducing the named restriction sites. The amplified fragment as well as the vector pRS-ScTEF1p-lacZ was digested with *PstI* and *XbaI*, separating the lacZ from the backbone vector. The following ligation placed *ScSFL1* behind the *ScTEF1p* in the plasmid, which was then used to transform *Agleu2* The transformants did not show any significant altered phenotype.

4.4. Localisation studies of AgSfl1 by using a GFP label

In *Candida albicans* it has been shown that Sfl1 localizes to the nucleus in yeast and hyphal cells (Bauer, J. and Wendland, J. 2007). The nuclear localisation domain of AgSfl1 makes it likely to detect the protein in nuclei in this organism as well. To study the localisation of this protein a C-terminal fusion with GFP (S65T) was chosen.
The ORF of *AgSFL1* with a length of 2088 bp was amplified from genomic DNA including 664 bp of the 5'UTR containing the gene promoter and 119 bp of the 3'UTR using primers 5'-AgSFL1 and 3'-AgSFL1-SacI, an annealing temperature of 52°C and a synthesis time of 3 min. The PCR fragment and the plasmid pRS415, bearing a *kanMX* resistance marker, were then digested with *PstI* and *SacI* and gel purified followed by ligation of the fragment into the plasmid and transformation in *E.coli* DH5a resulting in the plasmid pRS AgSFL1cc (#C327). The plasmids were extracted from resistant cells, and the correct insertion was verified by analytical digest and sequencing. The GFP and *kanMX* resistance marker gene were amplified by PCR using primers S1-GFP-AgSFL1 and S2-GFP-AgSFL1 and the plasmid pGUG as template. The PCR conditions included 10 cycles with an annealing temperature of 48°C and an elongation time of 3 min followed by 25 cycles with an annealing temperature of 52°C and the same elongation time. The product was gel purified as well and used together with the plasmid pRS AgSFL1cc (#C327) for homologous recombination in the yeast strain BY4743. This could be accomplished because by use of S1 and S2 primer a homologous sequence of 50 bp to the *SFL1* 3'UTR was introduced into the GFP containing PCR product (Fig. 30). The correct insertion of the fluorescence marker was verified by PCR and the

plasmid was then transformed into *E.coli* DH5á for amplification. After extraction, the plasmid was sequenced and used for transformation of *A. gossypii*

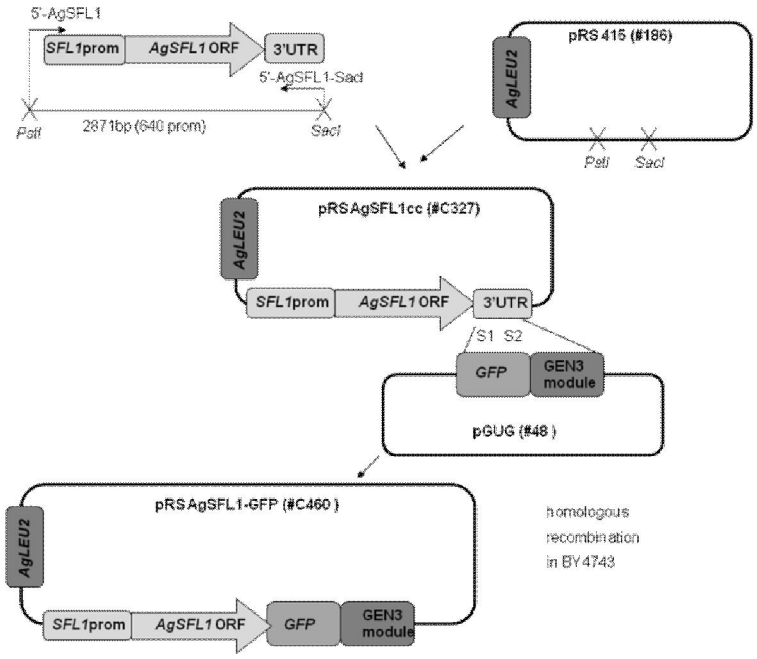


Figure 30. Construction scheme of pRSAgSFL1-GFP. The ORF of *AgSFL1* including 664 bp of the 5'UTR containing the gene promoter and 119 bp of the 3'UTR was PCR amplified introducing *PstI* and *SacI* restriction sites. The fragment was then inserted in pRS415 using the same sites. GFP and *GEN3* module were amplified by PCR introducing homologous regions to the 3'UTR of *AgSFL1* that led to homologous recombination in BY4743, placing the GFP at the 3'region of *AgSFL1*

4.4.1. Localisation in *A. gossypii*

For transformation of *A. gossypii* the strain *Agsfl1* was used. In this strain without an endogenous copy of *SFL1* all expression comes only from the plasmid born gene. The presence of the plasmid was verified by PCR. The geneticin resistance could not be used because in this strain it had already been used for deletion of the *SFL1* gene. The strain was selected on CSM-leu instead because pRS 415 contains an *ScLEU2* gene that can complement the *LEU2* auxotrophy of the mutant. For the incubation 20 mg/l adenin, 1 g/l asparagin and 20 mg/l uracil were added. The images were taken after 5 days growth with passaging in liquid CSM-leu in a baffled flask shaking at 180 rpm at 30°C. 200 µl of cells were fixed by adding 500 µl 70% ethanol. 1 µl DAPI was added and pictures were taken after 5 min incubation at room temperature. The fluorescent images showed GFP signals that colocalised with signals from nuclear staining, which indicates a localisation of GFP fused AgSfl1 in the nucleus (Fig. 31) (microscope support A. Walther).

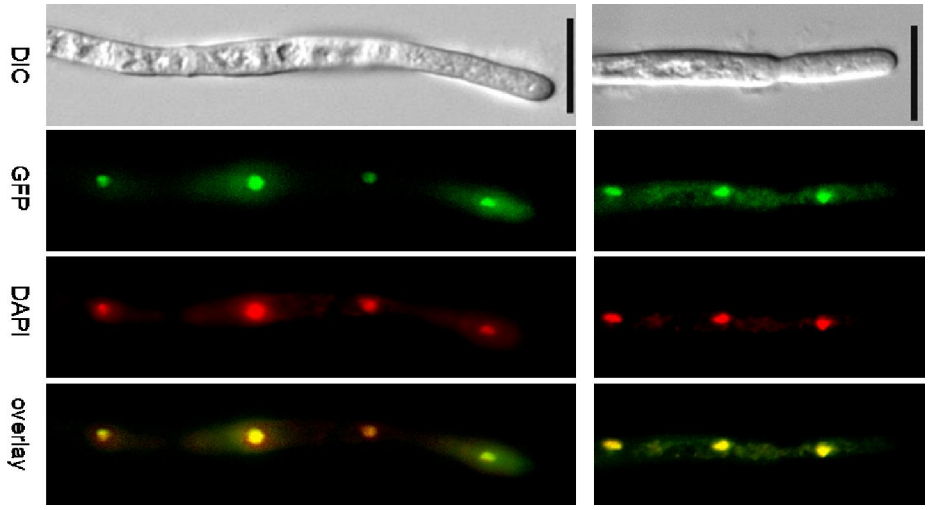


Figure 31. Fluorescence microscopy pictures of *Agsfl1* pRSAgSFL1-GFP. The images were taken after 5 days growth with passaging in liquid CSM-leu in a baffled flask shaking at 180 rpm at 30°C. 200 µl of cells were fixed by adding 500 µl 70% ethanol. 1 µl DAPI was added and pictures were taken after 5 min incubation at room temperature. The bar represents 10 µm.

4.4.2. Localisation in *S. cerevisiae*

Promoters of *A. gossypii* can be functional and control gene expression in *S. cerevisiae* (Wach, A. *et al.* 1994). Whether the *AgSFL1* promoter is functional in *S. cerevisiae* is not known. It would be of interest to see if AgSfl1 localises to nuclei in *S. cerevisiae* as well, since the nuclear localisation sequence differs only in one amino acid. The suppressor of flocculation should be situated in the nucleus in young cultures, since flocculation occurs only after exhaustion of nutrients.

BY4743 was transformed with the plasmid pRSAgSFL1-GFP and the geneticin resistance marker was used for selection. After one day growth in liquid YPD containing G418, the cells were fixed in 70% ethanol and 1 µl DAPI was added to 200 µl cell solution. Images were taken after 5 min incubation at room temperature (Fig. 32). The GFP signal colocalises only in a few cases with the nuclear stain. In most cases the GFP fluorescence shows multiple patches distributed in the cells, indicating that *AgSFL1* is localised cytoplasmic in *S. cerevisiae*

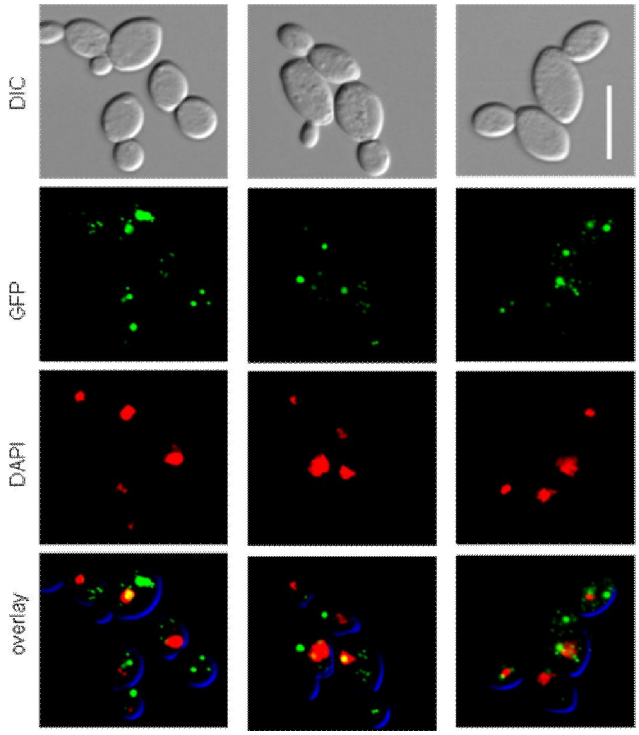


Figure 32. BY4743 pRSAgSFL1-GFP was grown in liquid YPD containing G418 for one day. The cells were fixed in 70% ethanol, and 1 µl DAPI was added to 200 µl cell solution. Images were taken after 5 min incubation at room temperature. The bar represents 10 µm. The GFP signal colocalises only in a few cases with the nuclear stain. In most cases the GFP fluorescence shows multiple patches distributed in the cells.

The cytoplasmic localisation of AgSfl1 in *S. cerevisiae* could indicate a non functional nuclear localisation sequence, or the protein gets clustered for discarding.

4.5. Expression studies of *A. gossypii* adhesin genes and transcriptional regulators using reporter gene constructs

To examine expression of flocculation genes and their transcriptional regulators in the different deletion strain backgrounds reporter gene constructs were generated. *Streptococcus thermophilus lacZ* was put under the regulation of promoters of genes of interest. The expression was analysed on colony level by spraying of X-gal (20 mg/ml in *N,N*-dimethylformamid) and by using â-galactosidase assays on mycelium grown on plates and in liquid culture (see Materials and methods).

To manifold the promoters of the *A. gossypii* genes, the intergenous region between the ORF of the gene of interest and the gene localised in front of it was amplified by PCR using KpnI-5'prom- and XhoI-3'prom-primers and genomic DNA as template. The

annealing temperature in the PCR was 52°C and the synthesis time 1.30 min. This resulted in amplified fragments of more than 500 bp for all promoters.

The amplified promoter fragments were gel purified and digested with *Kpn*I and *Xho*I as well as the plasmid pRS-AgTEFp-lacZ (#651). This was followed by another gel purification and ligation of the fragment into the plasmid. After transformation of *E. coli* DH5á, the amplified plasmids were verified by PCR and analytical digest. The constructs were meant for transformation of the deletion strains of adhesins and their regulators. For that reason a marker exchange had to be done, because the deletions were done using cassettes with either the *GEN3* module or the *kanMX* resistance gene. Therefore the marker genes in the reporter gene constructs were changed to nourseothricin resistance genes. The pFA NAT5 containing a nourseothricin resistance gene flanked by *S. cerevisiae TEF2* promoter and terminator was digested with *BstZ*171 and *Pac*I. This digest left 612 bp of the promoter and 64 bp of the terminator region to generate a homologous recombination after transformation of BY4741 with the restriction product together with the reporter gene constructs. This led to an exchange of the *GEN3*- with the *NAT1* resistance marker (Fig. 33). The resulting plasmids were amplified after transformation of *E. coli* DH5á, checked by analytical digest and sequencing, and then used for transformation.

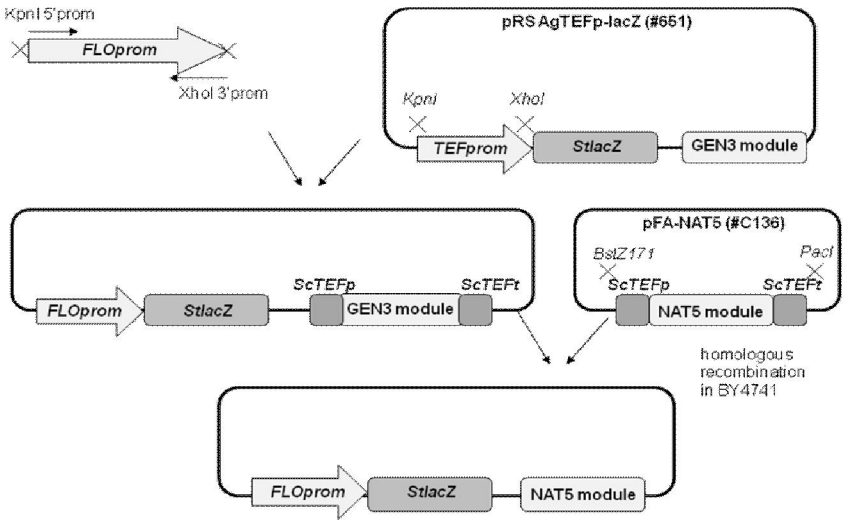


Figure 33. Construction scheme of pRSAgFLOp-lacZ constructs. Promoter regions of *FLO* genes and regulators were PCR amplified and restriction sites for *Kpn*I and *Xho*I were inserted. The same sites were used for replacing the *TEF* promoter in pRSAgTEFp-lacZ with the promoters. A marker exchange replaced the *GEN3*- with a *NAT5* module by homologous recombination in BY4741.

The strains *Agleu2*, *Agflo8*, *Agsfl1*, *Agfig2*, *Agtec1* and *Agste12* were transformed with the reporter gene plasmids. Growth assays on plate and in liquid culture and the following β-galactosidase assays were performed as described (see Materials and methods).

4.5.1. Expression studies on colony level

4.5.1.1. Expression studies on colony level in *Agleu2*

To see the reporter gene expression on colony level, 7 days old colonies on selective AFM clonNAT plates were sprayed with an X-gal solution, incubated at 37°C for 2 hours and pictures were taken. The situation in *Agleu2* is shown in Fig. 34.

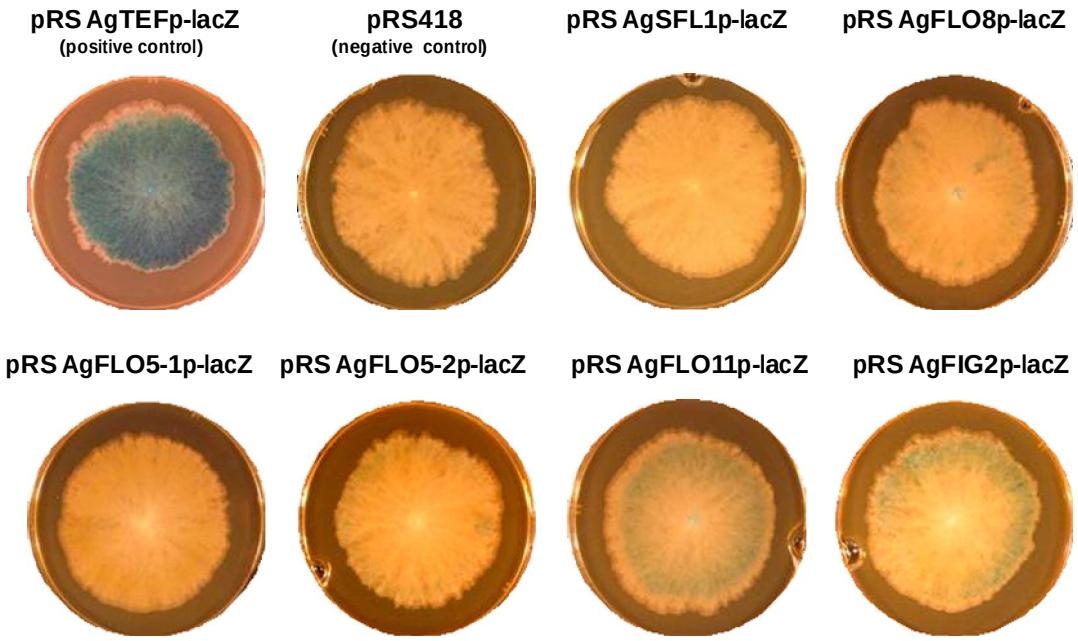


Figure 34. Reporter gene expression in *Agleu2* The positive control showed strong *lacZ*-expression and the negative control none. There was no colour visible for the *SFL1*- and the *FLO5-1*-promoter-driven construct. Slight blue staining occurred at the margin of the colony with the *FLO8*- and the *FLO5-2*-promoter containing construct, and stronger expression occurred in strains expressing *lacZ* under *FLO11*- and *FIG2* promoter regulation.

This first observation led to the speculation that *AgSFL1* and *AgFLO5-1* might not be expressed in *Agleu2* or only at a very low level. The Ashbya genome database (http://agd.vital-it.ch/index.html; Gattiker, A. *et al.* 2007) provides data to support the theory of non-expressed *AgFLO5-1* based on a DNA array data in early germlings.

Further it seems that *AgFLO8* and *AgFLO5-2* are expressed in low levels at the margin of a colony and *AgFLO11* and *AgFIG2* seems to be expressed in higher amounts.

4.5.1.2. Expression studies on colony level in other deletion mutants

In summary, in all other tested deletion mutants there was no *lacZ* expression under the control of *AgSFL1* and *AgFLO5-1* promoter detectable. The highest *lacZ* expression levels in all backgrounds were obtained under regulation of the *AgFIG2* promoter (Fig. 35).

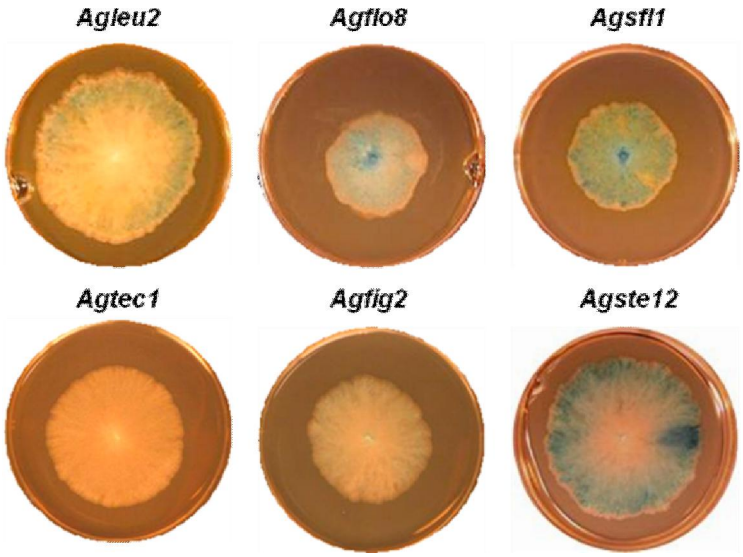


Figure 35. Reporter gene expression controlled by the *AgFIG2* promoter in different mutant backgrounds is shown. The *lacZ* expression regulated by *AgFIG2* promoter was strong in most deletion strains. *Agfig2* showed a weak expression, and *Agtec1* showed no *lacZ* expression at all.

This data indicates that *AgFIG2* expression was high in *Agleu2*, *Agflo8*, *Agsfl1* and *Agste12*. *Agfig2* showed a weaker expression and *Agtec1* no *AgFIG2* expression at all.
In *Agleu2* there was lacZ expression driven by the *AgFLO11* promoter visible at the age of 7 days. This reporter gene construct led to staining in the *Agsfl1*, *Agfig2*, *Agtec1* and *Agste12* backgrounds as well, but not in the *Agflo8* background (data not shown). Other reporter gene constructs in the adhesin and regulator deletion strains did lead to faint or no staining when treated with X-gal. For more exact information about the expression of reporter gene constructs, quantitative analyses were done.

4.5.2. Quantitative expression studies

For a quantitative analysis of the â-galactosidase activity, the transition of ONPG to o-nitrophenol and galactose was measured photometrically and Miller Units were calculated (see Materials and methods). To see if there is a correlation between expression and growing time, mycelium was harvested and analysed from plate- and liquid cultures after 24 h, 48 h and 72 h, respectively. Mycelium from a liquid culture that has been spread on plates was still not growing very fast after 24 h; this time was therefore considered as not sufficient to adapt to solid media and express relevant genes. After 72 h of growth, most strains started already to sporulate leading to a high standard deviation in calculations. This resulted in choosing mycelium of an age of 48 h for all further analysis. Table 6, Figure 36 and Figure 37 show the results of the â-galactosidase assay. According to standard deviations the threshold for significant expression was set around 10 Miller Units.

	FLOp-lacZ construct	*Agleu2*		*Agflo8*		*Agsfl1*		*Agtec1*		*Agfig2*		*Agste12*	
		Miller Units	Std	Miller Units	Std	Miller Units	Std	Miller Units	Std	Miller Units	Std	Miller Units	Std
48h plate	*FLO5 1*	2,0	1,1	1,8	2,5	1,8	1,5	0	1	0,2	0	0,2	0,2
	FLO5-2	9,7	1,5	14,0	2	24,8	3,1	10,4	5,2	8	1,6	10,1	2,0
	FLO8	12,5	1,1	18,2	3,1	18,8	3,2	10,5	3,8	3,8	0	8,8	0,1
	FLO11	8,8	2,8	0,1	1,8	7,6	1,5	7,4	1,2	6,1	0,7	2,6	0
	SFL1	0,5	0,4	0	0	0	0	1,4	1,3	1,2	0,2	0,9	0,5
	FIG2	48,2	3,7	68,4	13,5	136,4	5,4	0,6	0,5	13,4	0,4	45,6	5,5
48h liquid	*FLO5 1*	1,3	0	0	0	1,2	0,8	1	0,1	0,5	0	0,7	0,1
	FLO5-2	32,1	3,8	8,4	0	10	1,1	13,9	3,2	4,5	0,1	3,0	0,3
	FLO8	13,0	7,1	18,6	2,1	13	2,1	15,6	6,1	5,3	0	16,2	0,5
	FLO11	4,5	1,5	3,7	0	7,7	0,9	5,8	1,8	4,3	1	4,2	0
	SFL1	3,8	1,8	1,5	0	1,5	0,7	2	0,8	0,2	0	1	0
	FIG2	52,4	22,7	51,4	2,1	91,8	3,6	0,2	0,1	26,6	0,6	64,1	0

Table 6. The table shows the results of the â-galactosidase assays of deletion strains carrying pRSAgFLOp-lacZ constructs that were grown either on selective AFM plate or in selective liquid full medium for 48 hours. Results in Miller Units and the standard deviation are shown. Amounts are relative, meaning the negative control was subtracted already.

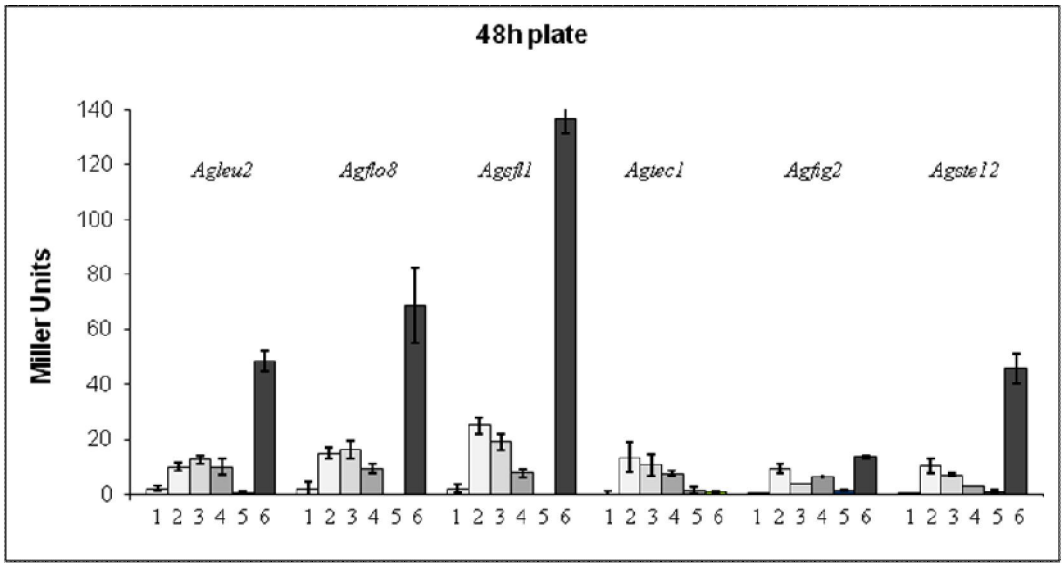


Figure 36. The diagram shows the results of the â-galactosidase assay from mycelium that has been grown for 48 hours on selective AFM plates. In all strain backgrounds *lacZ* expression under regulation of *FLO5-1* promoter and *SFL1* promoter was barely or not visible at all. Significant changes were visible in the *Agflo8* background, where *FIG2*-promoter driven *lacZ* expression was increased; in the *Agsfl1* background, where *FLO5-2*-, *FLO8*- and *FIG2*-promoter driven *lacZ* expression was increased and in the *Agtec1*and *Agfig2* background, where *FIG2*-promoter driven *lacZ* expression was absent or low, respectively.

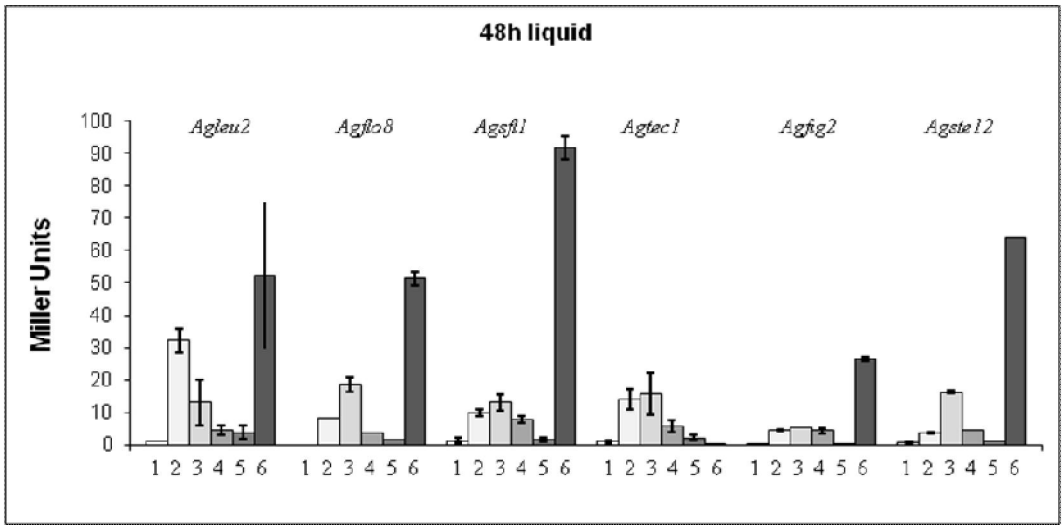


Figure 37. The diagram shows the results of the â-galactosidase assay from mycelium that has been grown for 48 hours in selective liquid full medium. In all strain backgrounds *FLO5-1* promoter and *SFL1* promoter controlled *lacZ* expression was barely or not detectable at all. Compared to the precursor strain, *FLO5-2*-promoter driven *lacZ* expression was lower in all

deletion strains. Significant changes were visible in the *Agsfl1* background, where *FIG2*-promoter driven *lacZ* expression was increased and in the *Agtec1*and *Agfig2* background, where *FIG2*-promoter driven lacZ expression was absent or low, respectively.

The significant changes in expression levels have been summarized in Fig. 38.

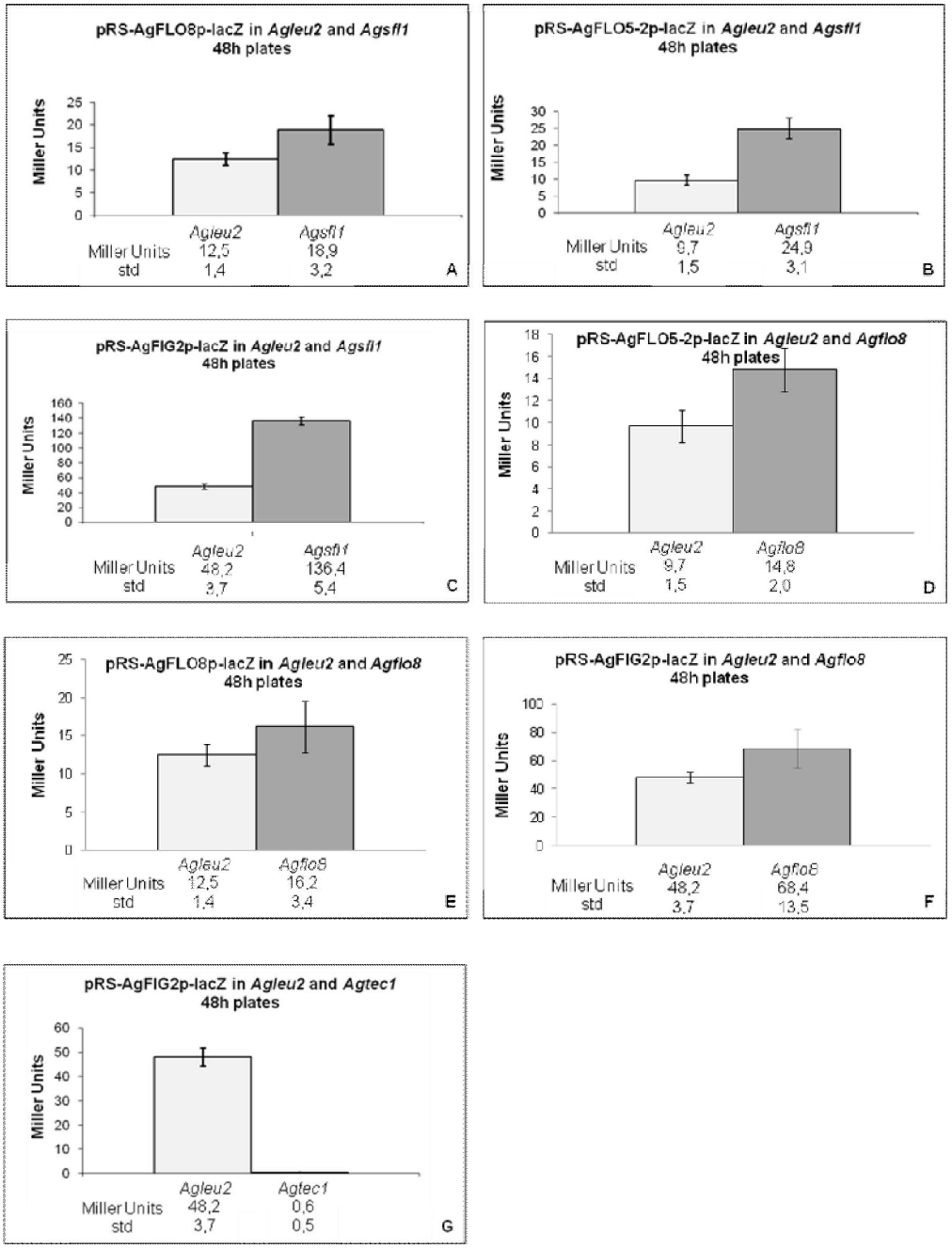


Figure 38. The diagrams show the significant changes of reporter gene expression in the given strains grown for 48 hours on selective AFM plates compared to the precursor strain *Agleu2* Shown in diagram A is the 1.5 fold upregulation of *FLO8* promoter regulated *lacZ* expression in *Agsfl1* compared to *Agleu2* B shows the 2.6 fold upregulation of *FLO5-2* promoter controlled *lacZ* expression in *Agsfl1*. In C the 2.8 fold stronger expression of *lacZ* driven by the *FIG2* promoter compared to the precursor strain is shown. Diagram D shows the 1.5 fold upregulation of *FLO5-2* promoter driven *lacZ* expression in *Agflo8*. Diagram E shows the 1.3 fold upregulation of *lacZ* expression under control of *FLO8* promoter in *Agflo8*. The *FIG2*-promoter

driven *lacZ* expression is 1.4 fold upregulated in *Agflo8* compared to *Agleu2* as shown in diagram F. Diagram G shows the complete loss of *lacZ* expression under *FIG2* promoter regulation in *Agtec1* compared to *Agleu2* and the same downregulation was observed in liquid culture.

In summary, in all strain background there was a very low *AgSFL1* and *AgFLO5-1* promoter driven *lacZ* expression detectable. As mentioned before, the array data from the Ashbya genome database support the assumption of absence of *AgFLO5-1* expression in the precursor strain. This is probably not due to a repression by AgSfl1 since the deletion of this factor led not to detectable amounts of the reporter gene under *FLO5-1* control. *AgSFL1* on the other hand is probably expressed in amounts below the detection level since its deletion led to upregulation of *lacZ* expression levels under control of three genes, being an indicator for its presence in the precursor strain.
Another result that was similar in all â-galactosidase assays was the absence of *AgFIG2* promoter controlled *lacZ* expression in an *Agtec1* strain and the reduced expression in an *Agfig2* strain. In all other strain backgrounds, *AgFIG2* promoter led to the highest expression levels. The absent expression in *Agtec1* is due to the absence of the main transcription factor, and *AgFIG2* promoter driven *lacZ* expression in *Agste12* is normal.
AgSfl1 seems to be a major negative regulator of *AgFIG2* expression, and in the *Agsfl1* deletion strain there is a derepression on plates and in liquid culture visible.
Further there seemed to be some differences between mycelium grown on plates and in liquid culture. Mycelium of the *Agflo8* mutant that was grown on plates but not in liquid medium showed an elevated *AgFIG2* promoter regulated *lacZ* expression and a slightly higher level with *AgFLO8* and *AgFLO5-2* promoters. This mimics the tendency in the *Agsfl1* mutant with elevated *lacZ* levels driven by these three promoters visible on plate cultures. This leads to the speculation that AgFlo8 might regulate the expression of *AgSFL1* and with that the regulation of *AgFIG2*, *AgFLO5-2* and *AgFLO8* itself. As mentioned before, in an *AgSFL1* deletion mutant there was in addition to the observed derepression of *AgFIG2* promoter regulated *lacZ* expression an elevated expression with *AgFLO5-2* and *AgFLO8* promoters compared to the precursor strain. This occurred only in mycelium grown on plates and not in liquid culture. In the promoters of these genes Sfl1 binding sites can be found, leading to the assumption that AgSfl1 acts as their transcriptional repressor.

4.6. Invasive growth of *Agtec1*

Köhler, T. *et al.* (2002) showed, that a deletion of *TEC1* in *S. cerevisiae* leads to a lack of invasive growth, which could be shown by a plate washing assay. To see if a similar phenotype can be observed in *A. gossypii* the *TEC1* deletion and the precursor strain were grown on AFM plates at 30°C for 3 days. The colonies were removed and the plate was rinsed with water followed by a further incubation at 30°C for one day (Fig. 39).

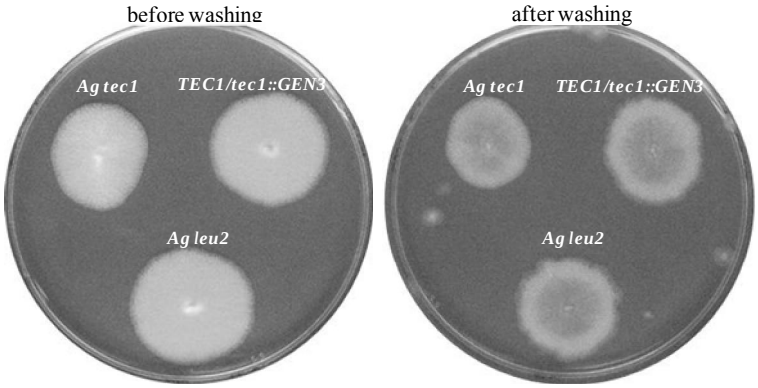


Figure 39. On AFM plates the *AgTEC1* deletion strain, the heterokaryon *TEC1/tec1::GEN3* and the precursor strain *Agleu2* were inoculated. After growth at 30°C for 3 days, colonies were removed, the plate was rinsed with water and further incubated at 30°C for one day. Colonies of *Agleu2*, the hetero- and the homokaryotic strain of *Agtec1* were regrowing in similar matters.

Compared to the behaviour of *Sctec1*, the *TEC1* deletion in *A. gossypii* does not lead to a loss of invasive growth.

4.7. Sporulation in *A. gossypii* deletion mutants

The *SFL1* deletion mutant seemed to sporulate abundantly compared to the precursor strain. This was also observed for *Agflo8* and *Agtec1* A microscopical analysis of spores of cultures, that has been grown for 5 days in CSM with myo-inositol at 30°C while shaking at 180 rpm, revealed no phenotypic difference compared to the precursor strain (Fig. 40).

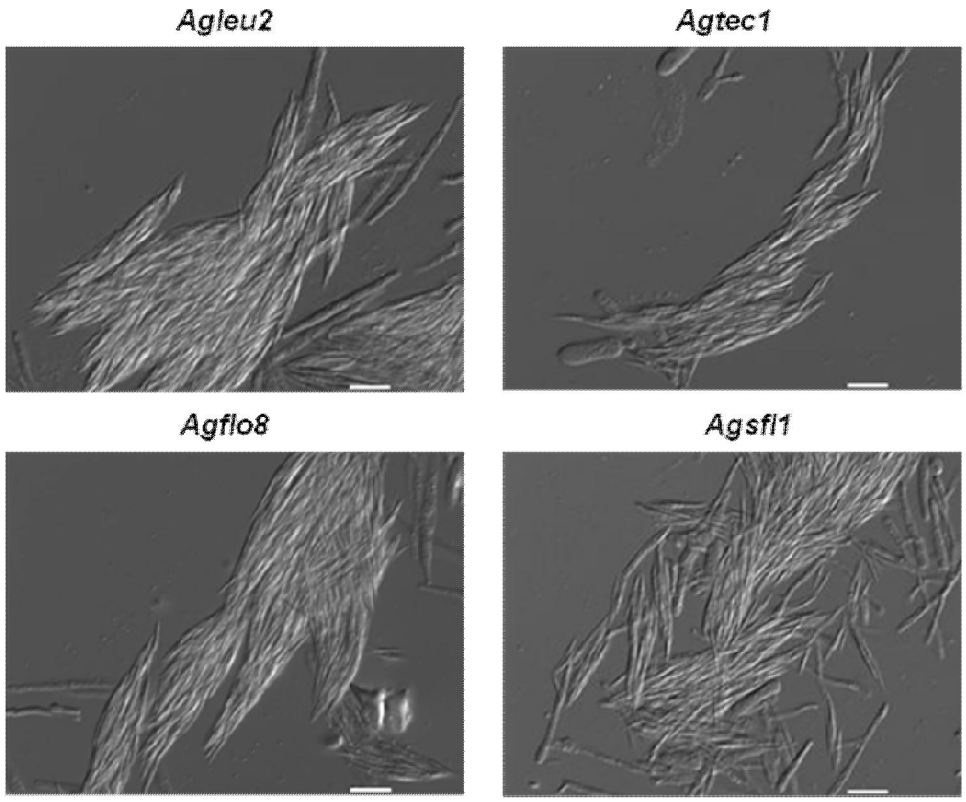
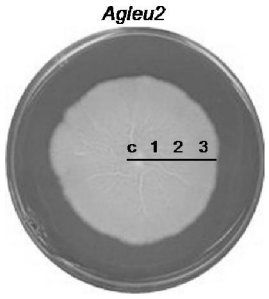


Figure 40. Spores of *Agleu2, Agtec1, Agflo8* and *Agsfl1* were prepared after 5 days of growth in CSM + myo-inositol, pictures were taken and the bar represents 20 µm. Spores of these mutants with a slight (in case of *Agtec1*) and a more severe growth defect (in case of *Agflo8* and *Agsfl1*) showed no difference to the precursor strain *Agleu2*

The phenotype of spores was not altered in this mutants but their amount was higher compared to *Agleu2* Unfortunately spores are tied together by their connecting tissue that cannot be removed, making it impossible to dilute them and count colony forming units. For that reason only an estimation about the amount of spores can be given. For this purpose colonies of *Agleu2, Agflo8, Agsfl1* and *Agtec1* on AFM plates were incubated at 30°C for 7 days before samples were taken from different zones of the diameter, and sporulation was microscopically analysed (Fig. 41). The result showed an abundant sporulation in the deletion strains compared to their precursor. Sporulation occurred, as for *Ashbya* typical, in the older parts of the colony.



	center	zone1	zone2	zone3
Agleu2	+	-	-	-
Agflo8	+++	++	+	-
Agsfl1	+++	++	+	-
Agtec1	+++	+++	+	-

Figure 41. Colonies of *Agflo8, Agsfl1, Agtec1* and their precursor strain *Agleu2* were grown on AFM at 30°C for 7 days and examined for sporulation. The colonies were divided in four concentric zones. A sample was taken from each zone, dissolved in water and microscopically analysed. The estimated quantity of spores found in the different zones in all examined strains is symbolized with "+" and "-" in the table. The slow growing mutants *Agflo8, Agsfl1* and *Agtec1* seemed to sporulate in larger amounts than the precursor strain *Agleu2*

4.8. Conservation of transcription factor binding sites between *A. gossypii* and *S. cerevisiae*

4.8.1. Reporter gene assay using pRSAgCTS2p-lacZ, plate phenotype

AgCTS2 (ACL166W) is the only chitinase in the genome of *A. gossypii* and it is the homologue to *ScCTS2*. It is specifically expressed in sporulating zones and therefore can be used as a reporter gene for spore producing mycelium (Dünkler, A. *et al.* 2008). The vector pRS417-AgCTS2p-lacZ (#656) contains a geneticin resistance marker, which cannot be used in the deletion strains carrying the same resistance gene. A marker exchange was done, performing a restriction digest of #656 and pFA-NAT5 (#C136) with the enzymes *BstZ*171 and *Pac*I, that set free the geneticin- and the nourseothricin resistance gene, respectively. The products were gel purified, and the nourseothricin resistance gene was ligated into the vector backbone. *E. coli* DH5á was transformed with the construct, and isolated plasmids were checked for their accuracy by analytical digest.

Agleu2, Agflo8, Agsfl1, Agfig2, Agtec1 and *Agste12* were transformed with the plasmids. To analyse the phenotype on solid medium, 100 µl of an 8 hour grown liquid culture were dropped at the centre of a selective AFM plate. After 2, 4 and 7 days of growth at 30°C, X-gal was sprayed on the colonies and pictures were taken after 2 hours further incubation at 37°C (Fig. 42).

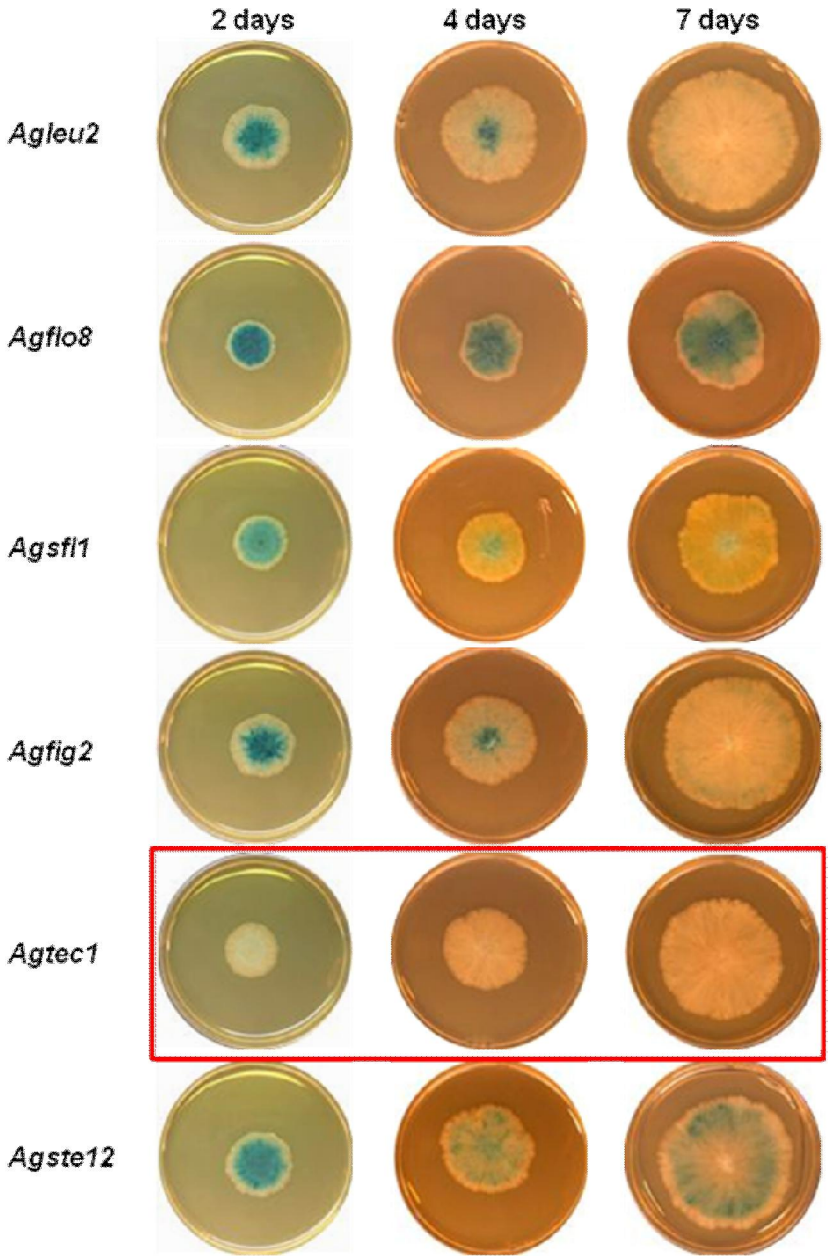


Figure 42. *Agleu2*, *Agflo8*, *Agsfl1*, *Agfig2*, *Agtec1* and *Agste12* were transformed with pRS417 AgCTS2p-lacZ-NAT5, grown on selective AFM clonNAT plates at 30°C for 2, 4 and 7 days before sprayed with X-gal. After further incubation at 37°C for 2 hours, pictures were taken. *LacZ* expression in *Agleu2*, *Agfig2* and to a stronger extend *Agste12* showed similar pattern, resulting in strong blue staining in the centre of the colony at an age of 2 days. The colour spread out to the margin over the following time period but had still the strongest stain in the centre. At the age of 7 days the colour remained visible at the margin of the colony. The expression in *Agflo8* followed the same pattern but was much stronger compared to the other strains and remained on the whole colony at the age of 7 days. This was also observable in *Agsfl1* but to a less extend. *AgCTS2* promoter driven *lacZ* expression in the *Agtec1* background on the other hand was completely absent.

All strains except *Agtec1* showed similar patterns in *AgCTS2* promoter regulated *lacZ* expression, indicating similar sporulation patterns, since *AgCTS2* is specifically

expressed in spore producing zones. Spores were produced in the oldest parts of the mycelium which is in the centre of a colony, spreading out towards the margin when colonies grew older. After a certain time, sporulation and *AgCTS2* expression ceased in the same order, leaving faint blue staining at the margin. The fact that there was stronger *lacZ* expression that covered the whole colony even at the age of 7 days in *Agsfl1* and *Agflo8,* indicates an elevated sporulation ongoing in the whole mycelium that does not cease after one weak in contrast to the other strains. As mentioned before, a higher sporulation was observed in these mutants. The fact that it does not cease like for the other strains, can be explained with the growth delay in *Agsfl1* and *Agflo8*. At the same age they are still in an earlier stadium of growth. There was no *lacZ* expression visible in *Agtec1* mutant although it is known to sporulate abundantly. Obviously AgTec1 seems to be a transcriptional regulator of *AgCTS2* expression, and two Tec1 binding sites could be identified in the promoter of *AgCTS2* Further this observation indicates, that *AgCTS2* is not involved in sporulation although its expression pattern colocalises with sporulation zones. In Dünkler, A. *et al.* (2008) it was shown, that the chitinase is not necessary for the release of spores from their surrounding tissue. There are obviously other ways, for example mechanical forces in shaking cultures or hyphal lysis in old mycelium, to ensure spore release.

4.8.2. Transcription factor binding sites in promoters of adhesins and regulators

LacZ expression driven by the *AgFIG2* promoter was completely absent in *Agtec1* mutants that were grown on plates or in liquid culture, whereas the expression was similar to precursor levels in the *Agste12* deletion mutant. Using the *S. cerevisiae* sequence, analysis of the *AgFIG2* promoter region showed several Tec1- but no Ste12 binding sites.

Binding sites for the transcriptional regulators Flo8, Sfl1, Tec1 and Ste12 are known for *S. cerevisiae* (see Introduction). A search for binding sites by similarity to the *S. cerevisiae* sequence was done in the promoters of the flocculation genes and their transcriptional regulators. Tec1- and Ste12 binding sites are listed in the table 7 and table 8. The fact that sites for Flo8 and Sfl1 are bipartite and their orientation and conservation in *A. gossypii* is unknown leads to identification of several of each in all promoters tested. If this is true needs to be analyzed in further experiments.

	Tec1 binding site, consensus: CATTC(C/YY)		
	sequence	orientation	distance from ORF
AgFLO5-1	CATTCAT	>	755 bp
AgFLO5-2	CATTCGA	>	11 bp
AgFLO11	0		
AgFIG2	CATTCTT	>	471 bp
	CATTCC	>	423 bp
	CATTCTT	>	275 bp
	CATTCGG	<	35 bp
AgFLO8	CATTCGG	>	456 bp
AgSFL1	CATTCAC	<	51 bp
AgTEC1	0		
AgSTE12	CATTCC	>	177 bp

Table 7. The table shows Tec1 binding sites in promoters of *A. gossypii* flocculation genes and their regulators, respectively. The consensus sequence is according to ScTec1. The actual sequence, the orientation in respect to the ORF and the distance from it is shown.

	Ste12 binding site, consensus: TGAAAC(A/G)		
	sequence	orientation	distance from ORF
AgFLO5-1	0		
AgFLO5-2	0		
AgFLO11	TGAAACT	>	455 bp
	TGAAACG	>	320 bp
AgFIG2	0		
AgFLO8	TGAAACA	>	725 bp
AgSFL1	TGAAACC	>	350 bp
AgTEC1	0		
AgSTE12	TGAAACG	<	312 bp
	TGAAACA	>	301 bp
	TGAAACT	>	287 bp
	TGAAACT	<	119 bp

Table 8. The table shows Ste12 binding sites in promoters of *A. gossypii* flocculation genes and their regulators, respectively. The consensus sequence is according to ScSte12. The actual sequence, the orientation in respect to the ORF and the distance from it is shown.

Although other adhesin genes apart from *AgFIG2*, from which only *AgFLO5-2* and *AgFLO8* are expressed above the threshold, show Tec1 binding sites in their promoter, there is no difference in expression between the *Agtec1* mutant and the precursor strain visible. This could either be due to their single appearance in the promoter or because of their difference in the last base pair compared to the consensus sequence. In further experiments the expression of *AgSTE12* in the *Agtec1* background should be observed, since this gene contains a single Tec1 binding site with the ScTec1 consensus sequence

in its promoter. Binding site studies including partial deletions of promoters would reveal further insight in *A. gossypii* gene regulation.

There are two Sfl1 binding sites in the promoter of *AgFIG2*. The first one is AGAA-n-TTCTT (n=121) about 276 bp from the *AgFIG2* ORF and the second one is AGAA-n-TTCTT (n=154) about 171 bp from the ORF. Work for deletion of these binding sites and analyzing reporter gene expression driven by the truncated promoter is in progress. Since AgSfl1 has been shown to be the negative regulator of *FIG2* expression, the reporter gene expression should be elevated after transformation in *Agleu2*

4.8.3. Analysis of Tec1 transcription factor binding site conservation

The previous experiment showed the missing *AgCTS2* promoter controlled *lacZ* expression in *Agtec1*, indicating the major role as a transcriptional activator.
For the quantitative analysis of the *lacZ* expression, β-galactosidase assays were performed. Since *AgCTS2* is expressed in spore producing mycelia, sporulation cultures were prepared. 20 ml CSM with myo-inositol were inoculated with 1 ml cell suspension from an 8 hour grown culture. The inoculated baffled flasks were incubated at 30°C and 180 rpm for 24, 48 or 72 hours, respectively, before harvested, and Miller Units were calculated (Fig. 43).

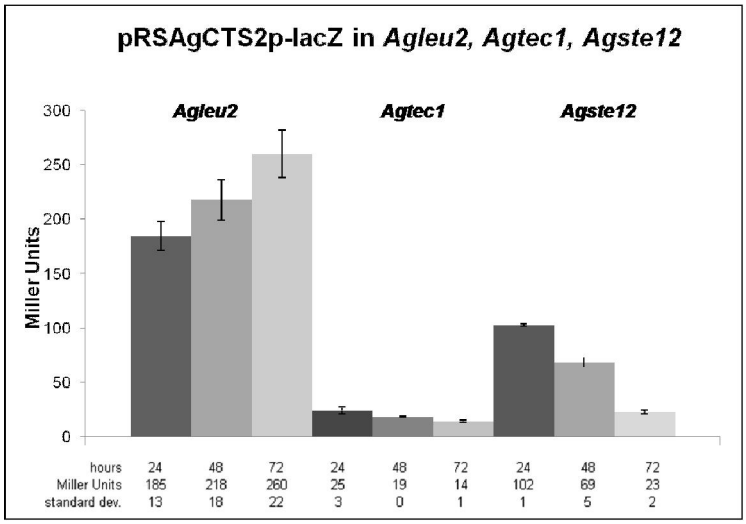


Figure 43. The *AgCTS2* promoter driven *lacZ* expression was high in *Agleu2* at all three time points in increasing manner. *Agtec1* in contrast showed a very low expression with a slight decrease over time. In the *Agste12* mutant the expression was lower than in the precursor strain and showed decrease over time. Miller Units and standard deviations are shown.

By comparison to the *S. cerevisiae* genome, 2 binding sites for Tec1 could be found in the promoter of *AgCTS2* at position –145 with the sequence CATTCAT, inverse oriented, and at position –78 with the sequence CATTCTC, also inverse oriented. The consensus sequence for the ScTec1 is CATTCYY. This indicates that AgTec1 seems to be a major inducer of *AgCTS2* expression and ScTec1 binding sites seems to be conserved in *A. gossypii*

The analysis of the *AgCTS2* promoter showed three putative binding sites for Ste12, although in comparison to the ScSte12 binding site (A)TGAAAC(A) the sequences showed some differences. There were two in inverse orientation at position –132 with the sequence TTGAAATA and at position –73 with the sequence TTAAAACA. Another one could be found at position –47 with the sequence TTGAAATC in forward orientation. Since they bear some similarity to the yeast consensus sequence it is likely that they could be functional but maybe not at full extent. This could be an indicator for the reduced but not absent *AgCTS2* promoter regulated *lacZ* expression in *Agste12*

4.8.4. Analysis of Tec1 transcription factor binding sites in the genome of *A. gossypii*

The whole genome of *A. gossypii* was searched for Tec1 transcription factor binding sites. The known sequence for *S. cerevisiae* is CATTC(C/YY). Since the consensus sequence for *A. gossypii* is not known, the sequence CATTC(C/NN) was used for the search. Orientation of sites could be either forward or reverse since both are possible in *S. cerevisiae*. Tec1 binding site specificities are shown in table 9 and table 10.

Chr(Tec1 regulated genes)	sequences of Tec1 binding sites													
	cattcc		cattctc		cattctt		cattcaa		cattcag		cattcga		cattcgg	
I (142)	51	35,9%	8	5,6%	19	13,4%	9	6,3%	11	7,7%	10	7,0%	8	5,6%
II (138)	34	24,6%	11	8,0%	8	5,8%	14	10,1%	8	5,8%	11	8,0%	9	6,5%
III (173)	58	33,5%	15	8,7%	12	6,9%	9	5,2%	9	5,2%	10	5,8%	7	4,0%
IV (318)	93	29,2%	25	7,9%	34	10,7%	28	8,8%	20	6,3%	16	5,0%	20	6,3%
V (306)	100	32,7%	17	5,6%	17	5,6%	17	5,6%	19	6,2%	16	5,2%	18	5,9%
VI (355)	97	27,3%	21	5,9%	27	7,6%	21	5,9%	25	7,0%	16	4,5%	19	5,4%
VII (305)	97	31,8%	10	3,3%	29	9,5%	28	9,2%	19	6,2%	18	5,9%	26	8,5%
total 1737	530	30,5%	107	6,2%	146	8,4%	126	7,3%	111	6,4%	97	5,6%	107	6,2%

Chr(Tec1 regulated genes)	sequences of Tec1 binding sites											
	cattcac		cattcgc		cattcat		cattcgt		cattcta		cattctg	
I (142)	6	4,2%	11	7,7%	10	7,0%	12	8,5%	12	8,5%	14	9,9%
II (138)	17	12,3%	8	5,8%	18	13,0%	9	6,5%	6	4,3%	13	9,4%
III (173)	14	8,1%	16	9,2%	16	9,2%	16	9,2%	11	6,4%	24	13,9%
IV (318)	15	4,7%	24	7,5%	28	8,8%	16	5,0%	23	7,2%	33	10,4%
V (306)	14	4,6%	25	8,2%	16	5,2%	17	5,6%	34	11,1%	26	8,5%
VI (355)	24	6,8%	21	5,9%	30	8,5%	28	7,9%	26	7,3%	29	8,2%
VII (305)	16	5,2%	20	6,6%	25	8,2%	17	5,6%	30	9,8%	22	7,2%
total 1737	106	6,1%	125	7,2%	143	8,2%	115	6,6%	142	8,2%	161	9,3%

Table 9. The table shows the amount of Tec1 regulated genes for each chromosome on the left side and possible binding site sequences and their frequency of appearance. The bottom line shows the total amount of Tec1 controlled genes and the total amount and frequency of each binding site.

Chromosome I			Chromosome II			Chromosome III			Chromosome IV		
sites	genes		sites	genes		sites	genes		sites	genes	
1	81	57,0%	1	91	65,9%	1	109	63,0%	1	210	66,0%
2	41	28,9%	2	34	24,6%	2	43	24,9%	2	60	18,9%
3	12	8,5%	3	9	6,5%	3	18	10,4%	3	27	8,5%
4	3	2,1%	4	0	0,0%	4	3	1,7%	4	14	4,4%
>4	5	3,5%	>4	4	2,9%	>4	2	1,2%	>4	7	2,2%

Chromosome V			Chromosome VI			Chromosome VII			total		
sites	genes		sites	genes		sites	genes		sites	genes	
1	207	67,6%	1	249	70,1%	1	201	65,9%	1	1148	66,1%
2	67	21,9%	2	67	18,9%	2	71	23,3%	2	383	22,0%
3	18	5,9%	3	22	6,2%	3	19	6,2%	3	125	7,2%
4	10	3,3%	4	13	3,7%	4	4	1,3%	4	47	2,7%
>4	4	1,3%	>4	4	1,1%	>4	10	3,3%	>4	36	2,1%

Table 10. The tables show the amount of Tec1 binding sites in promoters of Tec1 regulated genes in *A. gossypii*Shown is the amount of genes of each chromosome that have one to four or more binding sites in their promoter and the total amount of Tec1 controlled genes with their binding sites.

Out of 4726 protein coding genes in the genome of *A. gossypii*,1737 (36.8%) show one or more Tec1 binding sites in their promoter when searched after CATTC(C/NN). Out of this Tec1 controlled genes around 30% showed the consensus sequence CATTCC.

The other sequences were present in about equal frequency with no significant enrichments in certain chromosomes. About 66% of all possible Tec1 regulated genes have only one binding site in their promoter. The frequency of more binding sites drops in parallel with their amount. By strictly acknowledging only the consensus sequence CATTC(C/YY) like for ScTec1, only 16.6% of all protein coding genes in *A. gossypii* are Tec1 regulated, and two third of these possess the binding site CATTCC in their promoter. This indicates clearly that conservation of consensus sequences needs to be examined further.

4.8.5. Analysis of Ste12 transcription factor binding site conservation

The binding sites for Tec1 seem to be conserved between *A. gossypii* and *S. cerevisiae* as shown in previous experiments. To analyse the conservation of Ste12 binding sites between both organisms a reporter gene construct consisting of *Streptococcus thermophilus lacZ* and the promoter of *AgRIB3* was used. *AgRIB3* encodes an enzyme catalysing the transformation of ribulose-5-phosphate to 3, 4 dihydroxy-2-butanone-4 phosphate in the riboflavin biosynthesis pathway. *AgRIB3* shows three putative Ste12 binding sites in its promoter. One is localised at position –287 and has the sequence CTGAAACT, one bears the sequence ATGGAACA and is localised at position –269, and the third one is situated at position –184 with the sequence GTGAAACA. All three are in forward orientation and are very similar to the *S. cerevisiae* Ste12 consensus sequence of (A)TGAAAC(A). The plasmid pRSAgRIB3p-lacZ was used for transformation of *Agleu2* and *Agste12* Quantitative analysis of the reporter gene expression was performed using β-galactosidase assays of mycelium grown on selective AFM plates and liquid cultures in selective full medium. Mycelium grown on solid medium was harvested after 24 or 48 hours at 30°C, respectively; mycelium from liquid cultures was harvested after 24, 48 or 72 hours at 30°C, respectively and Miller Units were calculated (Fig. 44 and Fig. 45).

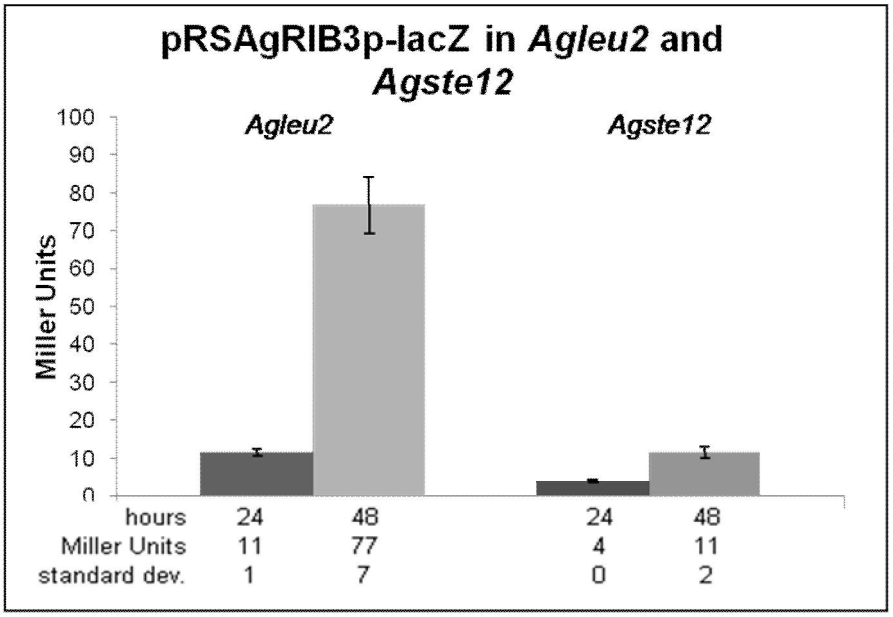


Figure 44. β-galactosidase assay of reporter gene expression in mycelium growing on solid medium. A high expression of the reporter gene construct was detectable in *Agleu2* with a strong increase over time. The expression in *Agste12* showed an increase as well but the total amount was much lower compared to the precursor strain. Miller Units and standard deviations are shown.

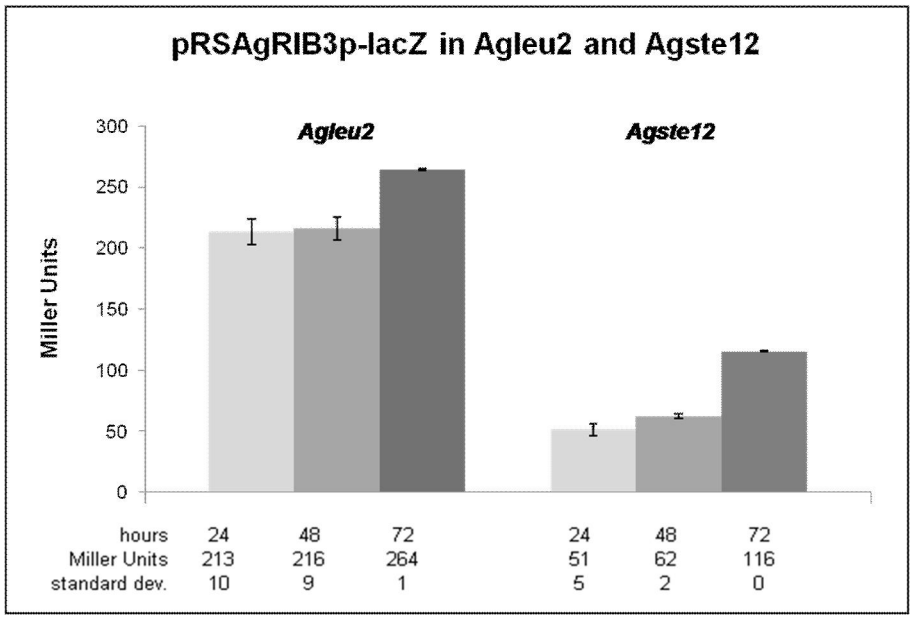


Figure 45. β-galactosidase assay of reporter gene expression in mycelium growing in liquid medium. *LacZ* expression controlled by the *AgRIB3* promoter was very high in *Agleu2* and showed a small increase over time. In *Agste12* there was an increase in *lacZ* expression over time as well but with much lower levels compared to *Agleu2* Miller Units and standard deviations are shown.

On solid and in liquid medium there was a high expression of the reporter gene construct detectable in *Agleu2* with an increase over time. The expression in *Agste12*

showed an increase during the examined time period as well, but the total amount was much lower compared to the precursor strain. This indicates that AgSte12 acts as an inducer of *AgRIB3* expression, and it makes the conservation of Ste12 binding sites between *A. gossypii* and *S. cerevisiae* likely. Interesting is the much lower amount of *lacZ* expression on plate cultures compared to liquid grown mycelia in general. The *AgRIB3* expression seems to start at a later time on plates than in liquid culture. This could indicate a delay in riboflavin production on plates compared to liquid media and has to be further examined.

5. Discussion

Ashbya gossypii belongs to the family *Saccharomycetaceae* and about 95% of its genes have a homologue in *Saccharomyces cerevisiae* (Ashby and Nowell 1926, Dietrich *et al.* 2004, Prillinger *et al.* 1997). The phenotypic differences between these two fungal species are huge, although there is a high genetic similarity. The phenotype of *A. gossypii* is mostly filamentous, whereas *Saccharomyces cerevisiae* occurs in a single cell state under normal conditions. *S. cerevisiae* can turn into a filamentous growth form under certain circumstances. Upon carbon depletion haploid yeast cells can grow filamentously, and diploids can form pseudohyphae during nitrogen depletion or starvation for amino acids (Roberts, R.L. and Fink, G.R. 1994; Gimeno *et al.* 1992; Braus *et al.* 2003). The expression of adhesion molecules is necessary to maintain the switch from a single cell state to pseudohyphae (Miki, B.L.A. *et al.* 1982; Lo, W.S. and Dranginis, A.M 1998). Adhesion molecules are cell wall glycoproteins, present on the surface of the cells of many fungi and mediate the attachment of cells with each other or with abiotic and biotic surfaces (Miki *et al.* 1982; Lo, W.S. and Dranginis, A.M 1998; Cormack *et al.* 1999). They have a modular construction. The N-terminal part maintains binding to certain peptides or sugar residues (Hoyer, L.L. *et al.* 1998; Kobayashi, O. *et al.* 1998). It is followed by a central domain consisting of serine- and threonine-rich peptide repeats, that undergo posttranslational glycosylation. The C-terminal domain bears a GPI anchor for securement of the protein on the cell surface by covalent binding to â-1, 6-glucans of the cell wall (Bony, M. *et al.* 1997). The N-terminal secretory sequence is cleaved off while transferring the protein through the secretory pathway (Hoyer, L.L. *et al.* 1998). In *Candida glabrata* adhesins are encoded by EPA (epithelial adhesion) genes and in *Candida albicans* by ALS (agglutinin-like sequence) genes (Hoyer, L.L. *et al.* 1995; Cormack *et al.* 1999). *S. cerevisiae* adhesion genes are called *FLO* genes and pheromone induced genes like *FIG2*. The *ScFLO* genes are *FLO1, FLO5, FLO9, FLO10* and *FLO11*.

5.1. Molecular analysis of *Ashbya gossypii* flocculation genes and regulators

Sequence comparison between the *S. cerevisiae* and the *A. gossypii* genes and proteins shows, that there are two Ag*FLO5* genes localised on the same chromosome in inverted orientation. *FLO5-2* is about the size of the *S. cerevisiae* homologue, whereas *FLO5-1*

is more than twice the size. They are both neither very similar towards the *S. cerevisiae* homologue nor to the *C. albicans CaYWP1* *A. gossypii* does not possess *ScFLO9* or *ScFLO10* homologues. The *ScFLO11* homologue in *A. gossypii* is about the same size but not very similar to the protein in *S. cerevisiae* or the *C. albicans* ortholog *CaEAP1* The same low similarity can be observed for the *FIG2*-like proteins between *S. cerevisiae* and *A. gossypii* Transcriptional regulators for the *FLO* gene expression in *S. cerevisiae* are among others Flo8 and Sfl1 in the cAMP-PKA pathway and Tec1 and Ste12 in the MAPK pathway (Fujita, A. *et al.* 1989; Kobayashi, O. *et al.* 1996; Liu, H *et al.* 1993). The similarity of Flo8 proteins between *S. cerevisiae, A. gossypii* and *C. albicans* is low, but the N-terminal part of Flo8 in all three organisms bears a LisH domain with higher similarity, suggested to be involved in microtubule dynamics and protein binding (Emes, R.D. and Ponting, C.P. 2001; Gerlitz G. *et al.* 2005). Sfl1 proteins of the three organisms are not very similar but bear a HSTF (heat shock transcription factor) homologous region at their N-terminus, which shows higher conservation and marks the proteins as transcription factors. The C-terminus of these domains contains a nuclear localisation sequence that enables the protein to enter the nucleus in order to influence gene expression (Cokol, M. *et al.* 2000). Tec1 proteins of *S. cerevisiae, A. gossypii* and *C. albicans* are not very similar towards each other, but their N-terminal parts contain a TEA domain that enables the binding of DNA and regulation of gene expression (Buerglin, T.R. 1991). The TEA domain is highly similar between the three organisms. Ste12 like proteins of these organisms have a STE domain with high conservation (Yuan, Y.O. *et al.* 1993).

In summary, adhesins of *S. cerevisiae* and *A. gossypii* do not possess a very high amount of similarity in general and are situated non-syntenically whereas their transcriptional regulators are localised at syntenic positions. Although the latter ones do not show high similarity either, their functional domains possess higher identity between *S. cerevisiae, A. gossypii* and also *C. albicans* The highest amount of conservation exists between STE domains of all three organisms (table 11 and table 12).

protein	*Ashbya gossypii*		*Saccharomyces cerevisiae*		*Candida albicans*		protein identity in %		
	systematic name	size in aa	systematic name	size in aa	systematic name	size in aa	*A. gossypii* *S. cerevisiae*	*A. gossypii* *C. albicans*	*S. cerevisiae* – *C. albicans*
Flo5	AFL095W	2535	YHR211W	1075	orf19.3618	533	32	29	23
	AFL092C	935					27	24	
Flo11	AEL023C	1485	YIR019C	1367	orf19.1401	653	28	24	29
Fig2	AFR711C	561	YCR089W	1609			28		
Flo8	AGL300C	939	YER109C	798	orf19.1093	792	14	24	13
Sfl1	AFR136C	695	YOR140W	766	orf19.454	805	25	21	18
Tec1	AER177W	791	YBR083W	486	orf19.5908	743	31	11	26
Ste12	ADR304W	655	YHR084W	688	orf19.4433	656	45	33	28

Table 11 The table shows information about adhesins and their transcriptional regulators in *A. gossypii, S. cerevisiae* and *C. albicans*, and percentages of protein identity between the organisms are indicated.

protein	domain	protein domain identity in %		
		A. gossypii - *S. cerevisiae*	*A. gossypii* - *C. albicans*	*S. cerevisiae* - *C. albicans*
Flo8	LisH domain	52	47	41
Sfl1	HSF domain	66	56	57
Tec1	TEA domain	70	49	62
Ste12	STE domain	87	80	74

Table 12 The table shows the identity of the indicated protein domains between *A. gossypii, S. cerevisiae* and *C. albicans*.

High similarity of domains indicates conserved functions in DNA binding and complex formation with other proteins, respectively. Adhesins in *S. cerevisiae*, *C. albicans* and *C. glabrata* have similar domain based structures and bear a GPI anchor for attachment to the cell surface. There are also other molecules with similar function but different structure in other organisms. *Schizosaccharomyces pombe* contains putative adhesins with a conserved tandem repeat similar to *C. albicans* Als proteins, but these molecules have no GPI anchor attachment signal. Instead they possess a GLEYA domain at their C-terminus, which enables ligand binding and is related to the lectin-like domain in *S. cerevisiae* Flo proteins. Ligand binding at the C-terminus of the protein has been observed in a majority of putative adhesins in the *Pezizomycotina* (Linder, T. and Gustafsson, C. M. 2008).

5.2. Deletion phenotype of *A. gossypii* adhesins and transcriptional regulators

The colony phenotype on full medium plates and on CSM of most of the strains was comparable with the precursor strain *Agleu2* Deletion mutants of the positive and negative regulator of the cAMP-PKA pathway, *FLO8* and *SFL1* respectively, showed a growth delay. The deletion of the transcriptional activator of the MAPK pathway, *TEC1*, resulted in slightly slower growth as well, whereas deletion of the other transcription factor of this pathway, *AgSTE12*, did not result in any phenotype. Attachment of deletion mutants to the plate surface was not impaired either. This observation is in contrast to *S. cerevisiae*, where deletions of flocculation genes lead to

absence of adhesion (Lo, W.S. and Dranginis, A.M. 1998 (*FLO11*), Kobayashi, O. et al. 1999 (*FLO8*) Köhler, T. *et al.* 2002 (*TEC1*)).

The same reduced growth phenotype after deletion of *SFL1* and *FLO8* as in *A. gossypii* could be observed in the isolate *Eremothecium gossypii* which is characterised by its strong riboflavin production and abundant sporulation. This result indicates that the growth phenotype is not strain specific but also occurs in other strain backgrounds. This and the fact that for all deletions independent mutants were examined, confirmed the absence of the respective gene as the reason for the observed phenotype.

The growth behaviour that occurred on AFM plates could also be observed in liquid culture, where only *Agflo8, Agsfl1* and to a lesser extent *Agtec1,* showed a reduced growth speed resulting in small mycelia balls in the medium.

As described before, young mycelia are characterised by lateral branching, whereas mature mycelium shows dichotomous tip growth. The microscopic analysis showed the fast outgrowth of hyphae of *Agleu2,* resulting in first lateral then Y-shaped branching of hyphae in growing zones. *Agsfl1* showed the same branching pattern but slower growth, resulting in more compact colonies with shorter outgrowing hyphae. After an additional day of incubation, *Agsfl1* hyphae behaved like earlier *Agleu2,* showing typical dichotomous branching. The phenotype observed for *Agsfl1* was similar in *Agflo8*.

The results of the reporter gene assays, with *StlacZ* regulated by promoters of *A. gossypii* adhesins and their transcriptional regulators, indicated similar adhesin expression profiles for *Agflo8* and *Agsfl1* due to an absence of Sfl1 in both mutants. In addition to the upregulated expression of *AgFLO5-2, AgFLO8* (for *Agsfl1*) and *AgFIG2* in these strains, it is possible, that other Sfl1 controlled genes are derepressed as well and lead to the observed phenotype. A hypothesis could be that the incorporation of the named adhesins into the cell wall or the higher expression of certain enzymes or components leads to a denser cell wall and therefore reduced endocytosis or growth speed. To examine changes in cell wall structure and stability, stainings and tests with wall interacting chemical components should follow. Array data could contribute to the examination of differently regulated genes in the indicated mutants.

Mature *A. gossypii* cultures on solid full medium form aerial hyphal aggregates similar to synnemata. These aggregates occur on the whole colony except for the outermost fringe. They seem to colocalize with sides of sporulation but are not necessary for it, because liquid grown mycelium sporulates without forming synnemata, and colonies on

minimal medium sporulate with barely developed aerial aggregates. Aerial hyphal aggregates of all deletion mutants showed similar amounts and sizes compared to the precursor strain, but those of *Agflo8* and *Agsfl1* were slightly smaller. This resembles the reduced growth of the mutants, since after more days of growth, synnemata reached sizes similar to *Agleu2* Although *A. gossypii* sporulates on CSM medium, there were barely aerial hyphal aggregations visible, making it unlikely for them to be involved in spore production. The size and amount relation of synnemata on CSM plates resembled those on AFM.

5.3. Sporulation in *AgFLO8, AgSFL1* and *AgTEC1* deletion mutants

Agsfl1, Agflo8 and *Agtec1* mutants seemed to sporulate in an abundant manner compared to *Agleu2* Unfortunately the sporulation amount could not be quantified, since spores tend to stick together due to their connecting tissue and cannot be separated in a manner that would allow counting of colony forming units. Microscopic analysis of samples from plate grown colonies showed elevated spore amounts for the named mutants. They were produced especially in the older mycelium in the centre of a colony, following therefore the pattern of *Agleu2* Microscopic analysis of spores revealed no phenotypic difference compared to the precursor strain.
Abundant sporulation of the three deletion mutants *Agsfl1, Agflo8* and *Agtec1* could be connected to reduced growth. Spore production requires resources and pathway components that could be missing for other cellular functions. Sporulation requires the fragmentation of hyphae, which could be a reason for slower outgrowth.

5.4. Phenotype of deletion mutants on AFM plates containing high sugar concentrations

The growth behaviour of deletion mutants was also tested on AFM containing 1 M glucose or fructose, respectively. This resembles more the natural environment of *Ashbya gossypii* as a plant pathogen on citrus fruits. The high sugar concentration led to slower growth of all strains. Single or double deletions of *FLO5-1* and *FLO5-2*, deletions of *FLO11, FIG2* or *STE12* behaved like the precursor strain. *Agflo8, Agsfl1* and to a less extend *Agtec1* showed reduced growth. Aerial hyphal aggregates of all deletion mutants showed similar amounts and sizes compared to the precursor strain.

Obvious was the smaller size of synnemata compared to colonies grown on AFM. This resembled the general slowed growth of all strains on high glucose concentrations.
Deletion strains *Agflo8, Agsfl1* and *Agtec1* did not show the same strength of growth restriction on colony morphology and synnemata size as on AFM, which could be due to possible insensitivity against high sugar concentrations. Furukawa, K. *et al.* (2009) described the negative regulative role of ScSfl1 on the aquaporine *AQY2* under hyperosmotic stress conditions independently of Flo8 and the HOG pathway. In *ScSFL1* deletion strains *AQY2* expression is elevated, and the *AQY2* overexpression phenotype shows strong surface attachment and invasive growth. This shows that in *S. cerevisiae* without Sfl1, the cell does not adapt to stress situations.
Galeote, V.A. *et al.* (2007) showed that ScSfl1 can also be an activator of gene expression, in that case of the heat shock protein encoding gene *HSP30* under certain stress conditions. A lack of ScSfl1 prevents from adaption to the stress situation. These examples show that ScSfl1 can act as a positive and negative regulator of gene expression, and its deletion can prevent the adaption to stress. This could be true for *A. gossypii* as well. A high sugar concentration resembles a hyperosmotic stress which leads to slower growth in *Agleu2* Mutants without functional Sfl1 partially overcome their growth delay, showing less sensitivity to the stress condition.
On the other hand when colony growth ceased, the sizes of precursor and deletion mutants converged. It could also be that growth ceased earlier on high sugar concentrations, leading to the observed convergence in colony size.

5.5. Effect of mannose on deletion mutants

The flocculins of *S. cerevisiae* are of the Flo1 type and repressible with mannose. According to Bayly, J.C. *et al.* (2005), 70 mM mannose leads to complete loss of flocculation. The phenotype of *Agleu2* and all adhesin deletion strains grown on AFM 70 mM mannose plates or in accordant liquid cultures resembled growth on AFM. Since mannose represses Flo1 type flocculation in *S. cerevisiae*, this impact could not be observed in *A. gossypii*. Either flocculins in this organism are repressible with other sugars, or a negative influence on flocculins does not lead to a phenotype, implying other proteins being involved in filamentous growth and attachment to surfaces.

5.6. Reporter gene assays using *lacZ* and promoters of adhesins and their transcriptional regulators

Under the tested conditions there was no phenotypic effect of deletion of flocculation genes *AgFLO5-1, AgFLO5-2* or *AgFLO11* or deletion of *AgFIG2* visible. A possible explanation could be the redundancy of these genes. Although a double deletion mutant for *AgFLO5-1* and *AgFLO5-2* did not show any phenotype, a null mutant of *AgFLO5-1, AgFLO5-2, AgFLO11* and maybe *AgFIG2* together could result in a phenotype. Guo, B. *et al.* (2000) showed that overexpression of *ScFIG2* could overcome the *ScFLO11* deletion defect, and overexpressed *ScFLO11* could compensate for the *ScFIG2* deletion. This functional compensation might be true for *A. gossypii* as well and might even lead to a redundancy depending on the regulation.

In order to get quantitative data of expression levels of adhesin genes and regulators, reporter gene assays have been established. *Streptococcus thermophilus lacZ* was put under the regulation of promoters of genes of interest.

The strains *Agleu2, Agflo8, Agsfl1, Agfig2, Agteca1* and *Agste12* were transformed with the reporter gene plasmids.

For a quantitative analysis of the â-galactosidase activity, the transition of ONPG to o-nitrophenol and galactose was measured photometrically, and Miller Units were calculated. Mycelium from a liquid culture that has been spread on plates was still not growing very fast after 24 hours. This time was therefore considered as not sufficient to adapt to solid media and express relevant genes. After 72 hours of growth, most strains started already to sporulate, leading to a high standard deviation in calculations. This resulted in choosing mycelium of an age of 48 hours for all further analysis.

In all strain backgrounds there was a very low *AgSFL1* and *AgFLO5-1* expression detectable, either by spraying of colonies with X-gal or in the â-galactosidase assay. That could be due to their absence or low amounts below detection level of this assay. The *Ashbya* genome database (http://agd.vital-it.ch/index.html; Gattiker, A. *et al.* 2007) provides data to support the theory of non-expressed *AgFLO5-1*, based on a DNA array data in early germlings of the wild type. This is probably not due to a repression by AgSfl1, since the deletion of this factor did not lead to derepression of the reporter gene under *FLO5-1* control. *AgSFL1* on the other hand is probably expressed in amounts below the detection level. Its deletion led to derepression of expression levels of three genes, being an indicator for its presence in the precursor strain. Posttranscriptional or

posttranslational regulation is an additional possibility. Phosphorylation of a protein for example occurs faster than transcription, and the cell can adapt quickly to changing environments. ScSfl1 and ScFlo8 are targets of phosphorylation by Tpk2, which regulates their binding to promoters (reviewed in Gancedo, J.M. 2001).

In defining 10 Miller Units as an expression threshold, detectable proteins in *Agleu2* were *AgFLO8* and *AgFIG2* on 48 hour old plates and additional to them *AgFLO5-2* in liquid culture. The with X-gal treated colonies on 7 day old plates indicated, that *AgFLO8* and *AgFLO5-2* were expressed in low levels at the margin of a colony, and *AgFLO11* and *AgFIG2* seemed to be expressed in higher amounts. This could point towards a time dependent adhesin expression. It seems that *AgFLO5-2* and *AgFLO11* become upregulated on plates over time. But for quantitative analysis, mycelium at that age cannot be used in â-galactosidase assays because of the high standard deviation caused by ongoing sporulation. Another examination method like RT PCR or western blot could be useful.

General *FLO* gene expression and induction over time is not altered in an *Agtec1* and *Agste12* deletion strain compared to the precursor strain. The exception is *AgFIG2*, which is not expressed in the absence of the transcriptional activator Tec1.

5.6.1. AgTec1 and *AgFIG2*

LacZ expression driven by the *AgFIG2* promoter was reduced in an *Agfig2* strain and absent in an *Agtec1* strain. In all other strain backgrounds *AgFIG2* was the adhesin with the highest expression level. The reduced expression in *Agfig2* seems to be due to a pleiotrophic effect of the deletion mutant, since expression levels of other reporter gene constructs were low as well. The absent expression in *Agtec1* is due to the absence of its transcription factor. In its promoter 4 potential AgTec1 binding sites could be found. This implies that AgTec1 is probably the main transcription factor for this gene, since *AgFIG2* expression in *Agste12* was normal. This shows a major difference compared to *S. cerevisiae*. In this organism, Ste12 is necessary for *FIG2* expression. ScFig2, which is expressed upon pheromone stimulation in mating conditions, is necessary for cell-fusion, agglutination and cell integrity during mating (Zhang, M. *et al.* 2002). The highly O- and N-glycosylated protein possesses an N-terminal secretion and C-terminal GPI anchor addition signal (Erdman, S.E. *et al.* 1998, Huang, G. *et al.* 2009). A MAPK cascade with G-protein coupled receptors Ste2 or Ste3 is involved in its expression

(reviewed in Gagiano, M. *et al.* 2002). According to Jue, C.K. and Lipke, P.N. (2002), disruption of *ScFIG2* shows different phenotypes depending on the strain background. In a S288C-derived strain absence of *FIG2* leads to increased agglutination, reduced mating frequency at 16°C and increased frequency at 30°C, whereas in the W303 background there was no growth effects and a minimal effect on mating efficiency. Reported are reduced viability under mating conditions, cell fusion defects and altered distribution of zygotic nuclei. These experiments did not show growth defects under non mating conditions, which is consistent with the described observation for *Agfig2*. Since there is no sexual cycle known for *A. gossypii*, there must be another regulation of the expression of *FIG2*.

AbaA of *A. nidulans*, which binds to AbaA-responsive elements that are identical to TCS, activates conidiophore development independently of the Ste12 similar protein SteA. This makes AbaA a regulator of asexual, whereas SteA is a regulator of sexual development in this organism (Vallim, M.A. *et al.* 2000). In *C. albicans* serum-induced hyphal growth and virulence are primarily regulated by CaTec1 independently of the Ste12-like protein CaCph1. CaCph1 regulates filamentous growth in response to nitrogen starvation (Liu, H. *et al.* 1994; Schweizer, A. *et al.* 2000). These examples show that in other organisms Tec1 and Ste12 homologues regulate different sets of genes, which seems to be the case in *A. gossypii* as well. And since Tec1 is preferentially a transcriptional activator of genes involved in asexual development in these organisms, its involvement in expression of *FIG2* in the asexual fungus *A. gossypii* makes sense.

AgSfl1 seems to be a major negative regulator of *AgFIG2*. In the *Agsfl1* deletion strain there was a 2.8 times higher expression of *AgFIG2* visible in â-galactosidase assays, and X-gal treatment showed its derepression on colony level as well. There are two Sfl1 binding sites in the promoter of *AgFIG2*. The first one is AGAA-n-TTCTT (n=121) about 276 bp from the *AgFIG2* ORF, and the second one is AGAA-n-TTCTT (n=154) about 171 bp from the ORF. Work for deletion of these binding sites and analyzing reporter gene expression driven by the truncated promoter is in progress. Since AgSfl1 has been shown to be the negative regulator of *FIG2* expression, the reporter gene expression should be elevated in *Agleu2*

5.6.2. AgFlo8

Mycelium of the *Agflo8* mutant that was grown on plates but not in liquid medium showed an 1.4 fold elevated *AgFIG2* promoter driven *lacZ* expression and a 1.3 fold or 1.5 fold higher level of *AgFLO8* and *AgFLO5-2* controlled expression, respectively. This mimics the tendency in the *Agsfl1* mutant with elevated levels of these three genes visible on plate cultures. Flo8 acts as a transcriptional activator in *S. cerevisiae* and is likely to have the same function in *A. gossypii* Since the expression levels of the reporter gene controlled by promoters of the named genes were higher in the *Agflo8* mutant compared to the precursor strain, it can be assumed that AgFlo8 regulates a negative regulator. Indeed in the promoter region of the transcriptional repressor *AgSFL1*, Flo8 binding sites could be identified. This leads to the speculation that AgFlo8 might regulate the expression of *AgSFL1* and with that the regulation of *AgFIG2*, *AgFLO5-2* and *AgFLO8* itself. It is possible, that without AgFlo8 there is no *AgSFL1* expression present.

Unfortunately, the Flo8 binding site, here referred to the sequence found in *S. cerevisiae*, consists of a bipartite sequence of 5 amino acids each separated by a region of variable length (Bester, M.C. *et al.* 2006). This sequence can therefore be found in many promoters in variable amounts. This and the fact that the conservation in *A. gossypii* is unknown, demand a further investigation of binding sites in this organism. The same is true for the binding site of Sfl1 (Conlan, R.S. and Tzamarias, D. 2001).

The data from the 7 day old colonies that were sprayed with X-gal supports the stronger expression of *AgFIG2* in an *Agflo8* strain. The expression was distributed over the entire surface of the colony, whereas the expression in the precursor strain was restricted to a marginal ring of the colony. Considering the probable time dependent expression of adhesins, as mentioned for *Agleu2*, there seems to occur a strong upregulation of *AgFIG2* expression in *Agflo8* over time. The higher expression seems to lead to a protein distribution all over the surface. On the other hand this appearance could also be due to the more compact colony compared to the precursor. There was no upregulation of *FLO11* expression over time, like it was for *Agleu2* This indicates the necessity of AgFlo8 as its transcriptional activator on solid medium.

5.6.3. AgSfl1

As mentioned before, in an *AgSFL1* deletion mutant, there was, in addition to the observed derepression of *AgFIG2*, a 2.6 fold elevated expression of *AgFLO5-2* and *AgFLO8* of about 1.5 fold compared to the precursor strain. This occurred only in mycelium grown on plates and not in liquid culture. In the promoters of these genes Sfl1 binding sites could be found, leading to the assumption AgSfl1 acts as their transcriptional repressor. *FLO8* expression is repressed by Sfl1 in *S. cerevisiae* as well as in *C. albicans* (Kim, T.S. *et al.* 2004; Bauer, J. and Wendland, J. 2007).
But the mentioned problems for AgFlo8, concerning amount and conservation of binding sites, are also true for AgSfl1, showing the need for further investigations.
The plates sprayed with X-gal showed very high levels for *AgFIG2* at the age of 7 days with the same distribution like for *Agflo8*. *AgFLO11* was expressed on 7 days old colonies. This resembles the induction of flocculin expression over time, like it was seen for *Agleu2*

5.6.4. *AgFLO11*

For *AgFLO11* there seemed to be a time dependent increase of expression. In all deletion backgrounds, except *Agflo8*, there was a *lacZ* expression regulated by the *AgFLO11* promoter observable after 7 days on solid medium. This suggests AgFlo8 to be the transcriptional activator for *AgFLO11*. As mentioned before there is no quantitative analysis using â-galactosidase assays with older mycelium possible due to ongoing sporulation. X-gal assays do not give quantitative data, thus further experiments using western blot or RT PCR to examine protein or RNA levels of adhesins need to be done.

5.6.5. Solid and liquid growth conditions

An interesting observation was the different behaviour of mycelium grown on plates and in liquid medium. In *Agleu2* there was a significant higher expression of *AgFLO5-2* in liquid culture compared to plate cultures. This suggests different requirements of flocculation genes depending on the growth condition. The observed derepression for plate grown cultures of *AgFLO5-2* and *AgFIG2* expression in *Agflo8* and *Agsfl1* mutants, was not to that extend present any more in liquid cultures. In *Agleu2* there was

a higher expression of *AgFLO5-2* in liquid culture but not for *Agsfl1* and *Agflo8*. As earlier mentioned, there could be a lack of AgSfl1 in *Agsfl1* and *Agflo8* mutant leading to mimicked phenotypes and expression levels. With this in mind, the data showed that the presence or absence of AgSfl1 does not have an effect on adhesin expression in liquid cultures. That means AgSfl1 is only functional on solid medium. Since the natural environment of *A. gossypii* is a solid surface of a cotton plant or citrus fruits, a tighter regulation of adhesins on solid medium makes sense. The fact that *Agleu2* shows a higher expression of *AgFLO5-2* in liquid compared to solid medium whereas other deletion mutants do not, could indicate involvement of another regulator whose function is disturbed in mutants lacking transcription factors of the cAMP-PKA or MAPK pathway.

To get more exact information about the amount of gene expression, the results of the reporter gene assay should be confirmed by RT-PCR concerning the adhesins and their transcriptional regulators. Protein analysis using western blot could reveal possible correlation between gene expression and protein occurrence and give a hint if posttranscriptional modification occurs. Further information about general transcript situation in deletion mutants of the transcription factors, for example with the help of arrays, would be helpful to understand the phenotypic differences like for *Agflo8* and *Agsfl1*.

A possible regulation scheme for adhesins in *A. gossypii* is shown in Fig. 46.

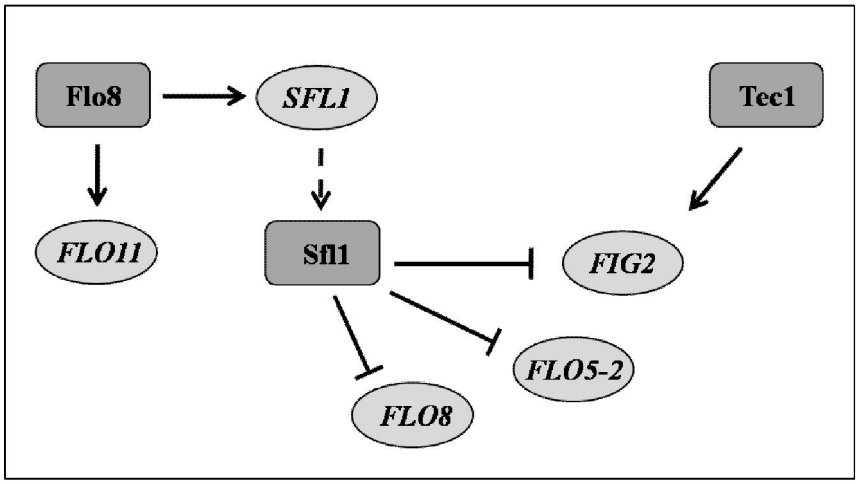


Figure 46. Shown is a possible regulation scheme for the expression of *A. gossypii* adhesins. Squared boxes indicate proteins, ovals indicate genes. Arrows symbolise positive influence in gene expression, dashed arrows translation and blocked lines repression of gene expression. The expression of *AgSFL1* could be positively regulated by AgFlo8 and AgSfl1 seems to repress transcription of *AgFLO8*, *AgFLO5-2* and *AgFIG2*. AgTec1 appears to be the major transcriptional activator of *AgFIG2*, and AgFlo8 seems to activate *AgFLO11* expression.

5.7. Other regulators and molecules involved in adhesion

Another explanation for the lack of phenotype of the examined mutants could be the involvement of other regulators or adhesin genes.
Gagiano, M. *et al.* 1999 described that the deletion of the transcriptional regulator *ScMSS11* results in complete absence of pseudohyphal growth that cannot be suppressed by overexpressing Ste12 and Msn1. Mss11, associated with the MAPK pathway, seems to be a key component in a complex that assists transcription factors in their regulation and forms complexes with Flo8 via their N-terminal LisH domains (Gagiano, M. *et al.* 1999; Gagiano, M. *et al.* 2003). The Ras-dependent transcription factor Msn1 acts MAPK independent.
MSS11 and *MSN1* can both be found in the genome of *A. gossypii*. Whereas Mss11 acts downstream of Flo8 in *S. cerevisiae* (van Dyk, D. *et al.* 2005) it seems to be located upstream of Flo8 in *C. albicans* (Su, C. *et al.* 2009).

In *S. cerevisiae* *FLO11* expression can be positively regulated by Phd1, Swi5 and Ash1 independently from the MAPK and the cAMP pathway (Gimeno, C.J. and Fink, G.R. 1994; Chandarlapaty, S. and Errede, B. 1998; Pan, X. and Heitman, J. 2000). A double mutation of *STE12* and *PHD1* causes complete loss of filaments in *S. cerevisiae* and *C. albicans*, whereas single mutations do not (Lo, H.J. *et al.* 1997). There is a *PHD1* homologue in *A. gossypii*, which could be an important regulator of flocculins.

Cln1 and Cln2 are important for the transcription of *FLO11* and pseudohyphal growth in *S. cerevisiae*. Grr1, the F-box protein in the SCF ubiquitin ligase complex, is involved in regulation of Cln1 and Cln2 in response to glucose- and amino acid availability. Low nitrogen levels result in downregulation of Cln3, a negative regulator of *FLO11* transcription. All three cyclins work independently from the Ste12-MAPK pathway. Further, a Cln1/2-like G1 cyclin is involved in hyphal formation of *C. albicans* in certain media (Barral, Y. *et al.* 1995; Loeb, J.D.J. *et al.* 1999a, b). *A. gossypii* possesses two G1 cyclins AgCln3 and AgCln1/2 and 3 B-type cyclins AgClb5/6, AgClb3/4 and AgClb1/2 (Hungerbuehler, A.K. *et al.* 2007). If the cyclins in *A. gossypii* have an influence on regulation of flocculation genes is not known.
In *S. cerevisiae* Ste12 binds and recruits the Swi/Snf chromatin remodelling complex to the promoter of *FLO11*, which enhances binding of Flo8 to the same promoter. Flo8 then recruits RNA polymerase II, and transcription can occur. For *Saccharomyces*

diastaticus it is postulated that in absence of glucose, Ste12 and Tec1 bind to the FRE in UAS2-1 and recruit the Swi/Snf complex to the promoter of *STA1* The *STA1* gene promoter has a high homology to the promoter of *FLO11* and a common regulation can be anticipated. The Swi/Snf complex promotes binding of Flo8 and Mss11 to UAS1-2 and lead to association of RNA polymerase II with the promoter (Kim, T.S. *et al.* 2004; Barrales, R.R. *et al.* 2008; Vinod, P.K.U. and Venkatesh, K.V. 2008). In *Candida albicans* overexpression of Swi/Snf bypasses the requirement of Ste12, Tec1 and Flo8 in invasive growth. In this organism Flo8 has a dual role as activator at high temperature and as repressor at low temperature (Su, C. *et al.*, 2009). Chromatin remodelling leads to better accessibility of DNA units and allows binding of transcription factors and polymerase. The involvement of this complex in adhesin gene expression in *A. gossypii* needs to be examined. A recruitment of the remodelling complex to promoters of adhesin genes could occur on the same or different ways in *A. gossypii* compared to *S. cerevisiae* and could involve different regulators.

Mao, X. *et al.* (2009) showed that in haploid *S. cerevisiae* strains, cAMP-PKA- and MAPK pathway are required for invasive growth and *FLO11* expression via the Swi/Snf complex. In diploid strains on the other hand, only the MAPK pathway with the transcriptional regulators Tec1 and Ste12 is required for the Swi/Snf recruitment and following *FLO11* expression. That result indicates that the ploidy determines the necessity of Flo8 as a transcriptional activator of the cAMP-PKA pathway. *A. gossypii* is haploid, and if this gene regulation mechanism is conserved, both pathways should be important for attachment and gene expression. This could emphasise the strong growth delay in *Agflo8* mutants and the weak delay in *Agtec1*

Yeast can adhere to surfaces without functional *FLO11* with the help of glucan and chitin in their cell wall. This occurs in a Swi5/Egt2/Cts2 dependent manner, where *EGT2* encodes an endoglucanase and *CTS2* an endochitinase (Pan, X. and Heitman, J. 2000). The cell walls of *S. cerevisiae* and *A. gossypii* are very similar; therefore it could be likely that a similar adhesion mechanism exists (Prillinger, H. *et al.* 1997).

A. gossypii encodes also other cell wall glycoproteins that could be responsible for surface attachment. In *S. cerevisiae* there are cell wall mannoproteins encoded by *CWP1* (YKL096W) and *CWP2* (YKL096W-A), which bear a GPI anchor and are necessary for

cell wall integrity, since a deletion of one or both lead to cell wall structure and permeability defects (Van der Vaart, J.M. *et al.* 1995). *A. gossypii* bears no homologue to *ScCWP2* but 7 copies of *CWP1* paralogs in its genome. Four of those are localised on chromosome II. This is ABR025C with 618 bp corresponding to 205 aa, ABR026C with 480 bp corresponding to 159 aa, ABR027C with 3396 bp leading to 1131 aa and ABR028C with a length of 1161 bp corresponding to 386 aa. Two *AgCWP1* genes can be found on chromosome III; ACR272C with 450 bp leading to a protein of 149 aa and ACR273W with 456 bp corresponding to 151 aa. The seventh *AgCWP1* gene ADL398C can be found on chromosome IV and has a length of 465 bp and a corresponding protein of 154 aa. The promoters of these genes were searched for Tec1 and Ste12 transcription factor binding sites according to their similarity to the *S. cerevisiae* binding sites shown in table 13. *A. gossypii* ABR025C, ABR038C and ADL398C seem not to have any involvement of these two MAPK cascade regulated transcription factors in their expression. These proteins could independently or together with the flocculation genes mediate attachment to surfaces in *A. gossypii* They could also contribute to adhesion in *Agtec1* or *Agste12* deletion mutants and need to be further examined.

Tec1 binding site, consensus: CATTC(C/YY)

	sequence	orientation	distance from ORF
YKL096W	CATTCTT	<	613 bp
YKL096W-A	CATTCGC	>	28 bp
ABR025C	0		
ABR026C	CATTCAC	>	118 bp
	CATTCAA	>	497 bp
ABR027C	CATTCGA	<	106 bp
	CATTCAC	<	510 bp
	CATTCTT	>	826 bp
	CATTCGA	<	893 bp
	CATTCTC	<	1413 bp
	CATTCTC	>	1443 bp
	CATTCGC	<	1725 bp
ABR028C	0		
ACR272C	CATTCTC	>	776 bp
ACR273W	CATTCTC	<	891 bp
ADL398C	0		

Ste12 binding site, consensus: TGAAAC(A/G)

	sequence	orientation	distance from ORF
YKL096W	TGAAACT	<	642 bp
YKL096W-A	0		
ABR025C	0		
ABR026C	0		
ABR027C	TGAAACA	<	604 bp
ABR028C	0		
ACR272C	TGAAACA	<	353 bp
ACR273W	TGAAACA	>	1314 bp
ADL398C	0		

Table 13 The tables show Tec1- and Ste12-binding sites in promoters of *S. cerevisiae CWP1* and *CWP2* and *A. gossypii CWP1* paralogs. The sequence, orientation and distance from the gene ORF are shown. Sequences were identified by similarity to *S. cerevisiae* binding sites.

5.8. Overexpression of *AgSFL1*

The deletion of the suppressor of flocculation led to a higher expression of flocculation genes, and a growth delay could be observed. An overexpression of *AgSFL1* on the other hand should lead to reduced expression of flocculation genes and possibly reduced

attachment. Since there was no reduction in adherence in single deletions of flocculation genes, the idea was that more than one gene suppressed by AgSfl1 gets downregulated in an *SFL1* overexpression mutant and leads to the speculated phenotype.

There was no colony phenotype visible between an *A. gossypii* strain overexpressing plasmid based *ScSFL1* and a reference strain. Since it is unclear if the heterologous expression could be blamed for that, plasmid based overexpression of *AgSFL1* was examined. This approach did not lead to a phenotype either. The unequal distribution of plasmids in the mycelium could be excluded as a possible explanation, since overexpression from the natural gene locus did not lead to a phenotype either. From the expression assay it is known that *AgSFL1* is expressed at low level but functional and responsible for repression of *AgFLO5-2, AgFLO8* and *AgFIG2*. The controlled genes are therefore suppressed below working level under normal growth conditions anyway, and *AgSFL1* overexpression does not lead to further changes.

5.9. Localisation studies of AgSfl1 by using a GFP label

5.9.1. Localisation in *A. gossypii*

In *Candida albicans* it has been shown that Sfl1 localizes to the nucleus in yeast and hyphal cells (Bauer, J. and Wendland, J. 2007). The nuclear localisation domain of AgSfl1 makes it likely to detect the protein in nuclei in this organism as well. To study the localisation of this protein, a C-terminal GFP fusion was chosen.

For transformation of *A. gossypii* the strain *Agsfl1* was used. In this strain without a functional copy of *SFL1*, all expression came only from the plasmid born gene. The fluorescent images showed signals that colocalised with signals from nuclear staining which indicates a localisation of AgSfl1-GFP in the nucleus. This confirms its nature as a transcription factor. To confirm the affiliation of AgSfl1 to the cAMP-PKA pathway in *A. gossypii*, cAMP could be supplied externally. With increasing cAMP levels there should be a higher activity of Tpk2, which leads to a large amount of phosphorylated Sfl1 that cannot bind to DNA any more. The localisation outside the nucleus could be a consequence.

Localisation studies of AgFlo8 and AgFig2 using a C-terminal fusion with GFP have been initiated.

5.9.2. Localisation in *S. cerevisiae*

For the localization study in *S. cerevisiae*, the plasmid bearing *AgSFL1* under control of its own promoter was used for transformation of BY4743. Promoters of *A. gossypii* can be functional and control gene expression in *S. cerevisiae* (Wach, A. *et al.* 1994). The suppressor of flocculation should be situated in the nucleus in young cultures, since flocculation occurs only after exhaustion of nutrients.

The GFP signal colocalised only in a few cases with the nuclear stain. In most cases the GFP fluorescence showed multiple patches distributed in the cells, indicating that *AgSFL1* is localised cytoplasmic in *S. cerevisiae*.

The *AgSFL1* promoter seems to be functional in *S. cerevisiae*, since *GFP* expression could be detected.

The cytoplasmic localisation of AgSfl1 in *S. cerevisiae* could indicate a non functional nuclear localisation sequence, since there is a difference of one amino acid between the two organisms. The third position of the sequence in *A. gossypii* is occupied by a serine and in *S. cerevisiae* by a histidine. The difference between a polar uncharged side chain for serine with a molecular mass of 105 g/mol and a charged aromatic side chain for histidine and the higher molecular mass of 155 g/mol could be responsible for the non function of the NLS of an *A. gossypii* protein in *S. cerevisiae*. Another explanation could be that the heterologous protein gets clustered for discarding maybe because of different posttranslational modifications in both organisms.

5.10. Examination of sporulation zones using reporter gene assay with pRSAgCTS2p-lacZ

AgCTS2 (ACL166W) is the only chitinase in the genome of *A. gossypii*, and it is the homologue to *ScCTS2*. It is specifically expressed in sporulating zones and therefore can be used as a reporter gene for spore producing mycelium (Dünkler, A. *et al.* 2008).

Agleu2, Agflo8, Agsfl1, Agfig2, Agtec1 and *Agste12* were transformed with a *lacZ* reporter gene construct including the *AgCTS2* promoter and examined on colony level.

All strains except *Agtec1* showed similar patterns in reporter gene expression, indicating similar sporulation patterns, since *AgCTS2* is specifically expressed in spore producing zones. Spores are produced in the oldest parts of the mycelium, which is in the centre of a colony. Production spreads out towards the margin, when colonies grow older. After a

certain time, sporulation and *AgCTS2* expression ceased in the same order, leaving faint blue staining at the margin. The fact that there was a stronger *lacZ* expression that covered the whole colony even at the age of 7 days in *Agsfl1* and *Agflo8,* indicates an elevated sporulation ongoing in the whole mycelium. Higher sporulation in these mutants had been observed earlier. Sporulation did not cease after one weak in contrast to the other strains. This can be explained with the growth delay in *Agsfl1* and *Agflo8*. At the same age they were still in an earlier stadium of growth. There was no *lacZ* expression visible in an *Agtec1* mutant, although it is known to sporulate abundantly. Obviously AgTec1 seems to be a transcriptional regulator of *AgCTS2* expression, and two Tec1 binding sites could be identified in the promoter of *AgCTS2* Further, this observation indicates that *AgCTS2* is not required for sporulation, although its expression pattern colocalises with sporulation zones. In Dünkler, A. *et al.* (2008) it was shown, that the chitinase is not necessary for the release of spores from their surrounding tissue. There are obviously other ways, for example mechanical forces in shaking cultures or hyphal lysis in old mycelium, to ensure spore release.

5.11. Conservation of transcription factor binding sites between *A. gossypii* and *S. cerevisiae*

5.11.1. Analysis of Tec1 transcription factor binding site conservation

The previous experiment showed the missing *AgCTS2* promoter controlled *lacZ* expression in *Agtec1*, indicating the major role as a transcriptional activator. Â-galactosidase assays from liquid cultures in the *Agleu2, Agtec1* and *Agste12* background, confirmed this first observation. The *AgCTS2* promoter driven *lacZ* expression was high in *Agleu2* and increased over a period of three days from 185 MU for 24 hours to 260 for 72 hours. *Agtec1* in contrast showed a very low expression with a slight decrease over time from 25 MU for one day to 14 MU for three days. In the *Agste12* mutant the expression was lower than in the precursor strain and showed decrease over time from 102 MU for 24 hours to 23 MU for 72 hours. The increased expression in *Agleu2* showed the correlation to increasing sporulation in this strain that has not ceased after three days. The fact that the colony pictures after X-gal treatment showed only a weak marginal stain at that time point, shows the less accuracy of this method and the need for the greater precision of the â-galactosidase assay. Further there can be a difference between mycelium grown in liquid culture and on plates.

The consensus sequence for the ScTec1 binding site is CATTCYY. By comparison to the *S. cerevisiae* genome, 2 binding sites for Tec1 could be found in the promoter of *AgCTS2*. One at position –145 with the sequence CATTCAT, inverse oriented, and one at position –78 with the sequence CATTCTC, also inverse oriented. This indicates that AgTec1 seems to be a major inducer of *AgCTS2* expression, and ScTec1 binding sites seems to be conserved in *A. gossypii*

The analysis of the *AgCTS2* promoter showed three putative binding sites for Ste12. In comparison to the ScSte12 binding site (A)TGAAAC(A), the sequences showed some differences. There were two in inverse orientation. One at position –132 with the sequence TTGAAATA and one at position –73 with the sequence TTAAAACA. Another one could be found at position –47 with the sequence TTGAAATC in forward orientation. Since they bear some similarity to the yeast consensus sequence, it is likely that they could be functional but maybe not at full extent. This could be a reason for the reduced but not absent *AgCTS2* promoter regulated *lacZ* expression in *Agste12*. The appearance of a decrease of the expression level over time in this mutant is in accordance with the stronger sporulation that ceases probably earlier compared to the precursor strain. Again the result from the plate assay, which showed a strong colour over three days, stands in contrast to the less expression level obtained in the â-galactosidase assay. This indicates the higher accuracy of the latter method. Further there can be a difference between mycelium grown on plates and in liquid cultures.

AbaA of *A. nidulans* binds to AbaA-responsive elements that are identical to TCS elements. The binding of sites differing in the fourth position occurs only in presence of high amounts AbaA (Adrianopoulos, A. and Timberlake, W.E. 1994). This suggests a tightly controlled binding. If this is the same in *A. gossypii* and if the binding sequence is exactly the same as for *S. cerevisiae*, needs to be further examined.

5.11.2. Analysis of Ste12 transcription factor binding site conservation

The binding sites for Tec1 seem to be conserved between *A. gossypii* and *S. cerevisiae* as shown in previous experiments. To analyse the conservation of Ste12 binding sites between both organisms, a reporter gene construct consisting of *Streptococcus thermophilus lacZ* and the promoter of *AgRIB3* was used. *AgRIB3* encodes an enzyme catalysing the transformation of ribulose-5-phosphate to 3, 4 dihydroxy-2-butanone-4 phosphate in the riboflavin biosynthesis pathway. Riboflavin production occurs in

parallel to sporulation, and the substance is thought to protect spores from UV light (Guilliermond *et al.* 1935; Stahmann, K.P. *et al.* 2001). The amount of enzyme should therefore correlate with the amount of riboflavin produced and further the level of sporulation. *AgRIB3* shows three putative Ste12 binding sites in its promoter. One is localised at position –287 and has the sequence CTGAAACT, one bears the sequence ATGGAACA and is localised at position –269 and the third one is situated at position –184 with the sequence GTGAAACA. All three are in forward orientation and are very similar to the *S. cerevisiae* Ste12 consensus sequence of (A)TGAAAC(A). The plasmid pRSAgRIB3p-lacZ was used for transformation of *Agleu2* and *Agste12*

On solid and in liquid medium there was a high expression of the reporter gene construct detectable in *Agleu2* with an increase over time. On plate culture the highest expression level was 77 MU after 48 hours and in liquid culture 264 MU after 72 hours. The expression in *Agste12* showed an increased expression during the examined time period as well, but the total amount was much lower compared to the precursor strain with 11 MU after 48 hours on plate or 116 MU after 72 hours in liquid culture. This indicates that AgSte12 acts as an inducer of *AgRIB3* expression, and it makes the conservation of Ste12 binding sites between *A. gossypii* and *S. cerevisiae* likely. Interesting is the much lower amount of *lacZ* expression on plate cultures compared to liquid grown mycelia in general. The expression seems to start at a later time on plates than in liquid culture. This could indicate a delay in riboflavin- and maybe also in spore production on plates compared to liquid media and has to be further examined. The fact that there is still an *AgRIB3* expression detectable in the *Agste12* background in liquid cultures indicates, that the Ste12 binding sites are maybe not conserved in full amount between *A. gossypii* and *S. cerevisiae*.

5.12. Transcription factor binding sites in promoters of adhesins and regulators

Binding sites for the transcriptional regulators Flo8, Sfl1, Tec1 and Ste12 are known for *S. cerevisiae*. A search for binding sites by similarity to the *S. cerevisiae* sequence was done in the promoters of the flocculation genes and their transcriptional regulators. The fact that sites for Flo8 and Sfl1 are bipartite and their orientation and conservation in *A. gossypii* is unknown, leads to identification of several of each in all promoters tested. If this is true needs to be analyzed in further experiments.

5.12.1. Ste12 binding sites

Analysis of the Ste12 binding sites (PRE) in promoters of adhesins and their regulators, revealed double occurrence in the promoter of *AgFLO11* where one is slightly different from the *S. cerevisiae* consensus sequence. The binding site occurs once for *AgFLO8* and once for *AgSFL1*, here with a slight difference to the consensus. In the *AgSTE12* promoter there are 4 binding sites, two different in one amino acid from the consensus. For *S. cerevisiae* it is known that single PRE are poorly bound by Ste12, but binding is enhanced by occurrence of multiple Ste12 binding sites (Dolan, J.W. *et al.* 1989; Yuan, Y.L. and Fields, S. 1991). In combination with a Tec1 binding site (TCS) a filamentation and invasion response element (FRE) arises. The combinatory binding of Ste12 and Tec1 to these elements leads to enhanced transcriptional activation (Baur, M. *et al.* 1997; Madhani, H.D. and Fink, G.R. 1997). The distance between these two binding sites is described with 14 bp. In the promoter of *AgFLO8*, *AgSFL1* and *AgSTE12* where there is one Tec1 binding site next to the mentioned Ste12 binding sites. The distance between these two is much larger, excluding the possibility of a functional FRE. The lack of Ste12 does not seem to have an influence on adhesin expression as seen in the morphology of the deletion mutant and the â-galactosidase assays. Since the Ste12 binding sites have been proven functional in *A. gossypii*, it could be their amount or sequence that differs slightly from the *S. cerevisiae* consensus that leads to malfunction in these genes. Further, it cannot be ruled out that other transcriptional regulators have influence on the expression of these genes. Since the promoter of *AgSTE12* is the only one with clustered PRE from which two share the *S. cerevisiae* consensus sequence, *AgSTE12* promoter regulated reporter gene expression should be examined in an *Agste12* deletion mutant. The *AgRIB3* promoter driven reporter gene expression in an *Agste12* background was not complete absent, although two of the three Ste12 binding sites share the *S. cerevisiae* consensus sequence and one differs in one amino acid. This implies either the need for a large amount of sites in promoters or shows that the conservation of these sites between the two organisms is not complete. In either way this could explain the lack of change in expression level of adhesins and their regulators in an *Agste12* mutant.

5.12.2. Tec1 binding sites

LacZ expression driven by the *AgFIG2* promoter was completely absent in *Agtec1* mutants, whereas the expression was similar to precursor levels in the *Agste12* deletion mutant. Analysis of the *AgFIG2* promoter region showed several Tec1- but no Ste12 binding sites. The consensus sequence CATTC(C/NN) was used as a search guideline due to a lack of knowledge about conservation between the *S. cerevisiae* consensus sequence CATTC(C/YY) and *A. gossypii*

Out of 4726 protein coding genes in the genome of *A. gossypii* 1737 (36.8%) show one or more Tec1 binding sites in their promoter when it was searched after CATTC(C/NN). Out of this Tec1 controlled genes, around 30% showed the consensus sequence CATTCC like it was described for *S. cerevisiae*. The other sequences were present in about equal frequency with no significant enrichments in certain chromosomes. This indicates conservation of this consensus between *S. cerevisiae* and *A. gossypii*. About 66% of all possible Tec1 regulated genes have only one binding site in their promoter. The frequency of more binding sites drops in parallel with their amount. By strictly acknowledging only the consensus sequence CATTC(C/YY) like for ScTec1, only 16.6% of all genes in *A. gossypii* are Tec1 regulated and two third of these possess the binding site CATTCC in their promoter. This indicates clearly that conservation of consensus sequences needs to be examined further.

Although other adhesin genes apart from *AgFIG2*, from which only *AgFLO5-2* and *AgFLO8* are expressed above the threshold, show Tec1 binding sites in their promoter, there is no difference between the *Agtec1* mutant and the precursor strain visible. This could either be due to their single appearance in the promoter or because of their difference in the last base pair compared to the consensus sequence. In contrast to *A. gossypii* *ScFLO8* and *ScFLO11* contain multiple individually arranged TCS elements in their promoter region that are not neighboured by a Ste12 binding site (Köhler, T. *et al.* 2002).

Binding site studies including partial deletions of promoters would reveal further insight in *A. gossypii* gene regulation as well as one hybrid experiments with AgTec1 and AgSte12 on the promoters of adhesin genes.

The promoters of *S. cerevisiae* *TEC1* and *AgTEC1* show a big difference concerning Ste12 and Tec1 binding sites. Whereas the *AgTEC1* promoter contains neither of them, the *ScTEC1* promoter contains 2 TCS and 4 PRE from which one FRE could arise with

a space of only 4 bp between the sites. According to Köhler, T. *et al.* (2002) *ScTEC1* is mostly independent of autoregulation though, but transcription is dependent on Ste12. The regulation of *AgTEC1* expression seems to be of other nature and needs to be further examined.

The promoter of *AgSTE12* shows one Tec1 binding site and 4 Ste12 binding sites as mentioned. *ScSTE12* promoter contains 4 Ste12 binding sites as well but no Tec1 binding site. A double deletion of *AgTEC1* and *AgSTE12* would be interesting to examine regarding expression levels of adhesins and transcriptional regulators. Construction of this mutant has been initiated, but deletion of either *AgTEC1* in *Agste12* background or deletion of *AgSTE12* in *Agtec1* background did not lead to viable homokaryons. If this suggests inviability of double deletion mutants needs to be further analysed.

Chou, S. *et al.* (2006) showed, that for the combinatorial regulation of ScSte12 together with ScTec1 on FRE, the N-terminal region of Ste12 from residue 1 to 215 is sufficient for the interaction with Tec1. The same region in AgSte12 shows 72% similarity with the *S. cerevisiae* protein (Fig. 47). Further, the authors concluded that residues 301 to 400 of ScTec1 are necessary for binding to ScSte12. This region in the *A. gossypii* protein shows only 30% similarity to ScTec1 (Fig. 48).

Figure 47. Protein alignment of the N-terminal 215 amino acids from AgSte12 and ScSte12.

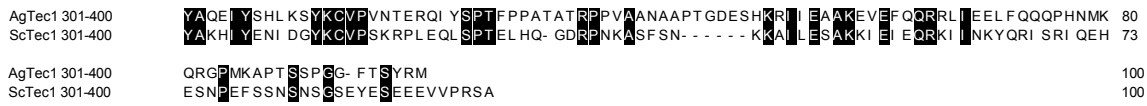


Figure 48. Protein alignment of amino acids 301 to 400 from AgTec1 and ScTec1.

If there is a protein complex of Ste12 and Tec1 in *A. gossypii* and if the same protein regions are involved needs to be further examined. A yeast two hybrid assay modified after Gietz, R.D. *et al.* (1997) has been initiated.

6. References

Adrianopoulos, A., Timberlake, W.E. (1991) ATTS, a new and conserved DNA binding domain. Plant Cell 3, 747-748.

Adrianopoulos, A., Timberlake, W.E. (1994) *Aspergillus nidulans* abaA gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol Cell Biol 14, 2503-2515.

Ashby, S.F., Nowell, W. (1926) The fungi of stigmatomycosis. Ann. Bot. 40, 69-84.

Bao, M.Z., Schwartz, M.A., Cantin, G.T., Ill, J.R.Y., Madhani, H.D. (2004) Pheromone-dependent destruction of the Tec1 transcription factor is required for MAP kinase signalling specificity in yeast. Cell 119, 991-1000.

Barral, Y., Jentsch, S., Mann, C. (1995) G1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast. Genes Dev 9, 399-409.

Barrales, R.R., Jimenez, J., Ibeas, J.I. (2008) Identification of novel activation mechanisms for FLO11 regulation in *Saccharomyces cerevisiae*. Genetics 178, 145-156.

Bauer, J., Wendland, J. (2007) *Candida albincans* Sfl1 suppresses flocculation and filamentation. Eukaryotic Cell 6 (10), 1736-1744.

Baur, M., Esch, R.K., Errede, B. (1997) Cooperative binding interactions required for function of the Ty1 sterile responsive element. Mo Cell Biol 17, 4330-4337.

Bayly, J.C., Douglas, L.M., Pretorius, I.S., Bauer, S.F., Dranginis, A.M. (2005) Characteristics of Flo11-dependent flocculation in *Saccharomyces cerevisiae* FEMS Yeast Research 5, 1151-1156.

Bester, M.C., Pretorius, I.S., Bauer, F.F. (2006) The regulation of *Saccharomyces cerevisiae FLO* gene expression and Ca^{2+}-dependent flocculation by Flo8p and Mss11p. Curr Genet 49, 375-383.

Bony, M., Thines-Sempoux, D., Barre, P., Bondin, B. (1997) Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. J. Bacteriol. 179, 4929-4936.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72, 248-254.

Braus, G.H., Grundmann, O., Brückner, S., Mösch, H.U. (2003) Amino acid starvation and Gcn4p regulate adhesive growth and *FLO11* gene expression in *Saccharomyces cerevisiae*. Mol. Biol. Cell. 14, 4272-4284.

Buerglin, T.R. (1991) The TEA domain: a novel, highly conserved DNA binding motif. Cell 66, 11-12.

Bumgarner, S.L., Dowell, R.D., Grisafi, P., Gifford, D.K., Fink, G.R. (2009) Toggle involving cis-interfering noncoding RNAs controls variegated gene expression in yeast. Proc Natl Acad Sci USA 106 (43), 18321-18326.

Campbell, S., Inambar, M., Rodrigues, V., Raghavan, K.V., Plazzolo, M., Chovnick, A. (1992) The scalloped gene encodes a novel, evolutionarily conserved transcription

factor required for sensory organ differentiation in *Drosophila* Genes Dev 5, 367-379.

Cao, F., Lane, S., Raniga, P.P., Lu, Y., Zhou, Z., Ramon, K., Chen, J., Liu, H. (2006) The Flo8 transcription factor is essential for hyphal development and virulence in *Candida albicans* Mol Biol Cell 17, 295-307.

Chandarlapaty, S., Errede, B. (1998) Ash1, a daughter cell-specific protein, is required for pseudohyphal growth of *Saccharomyces cerevisiae* Mol Cell Biol 18, 2884-2891.

Chou, S., Lane, S., Liu, H. (2006) Regulation of mating and filamentation genes by two distinct Ste12 complexes in *Saccharomyces cerevisiae* Mol Cell Biol 26 (13) 4794-4805.

Conlan, R.S., Tzamarias, D. (2001) Sfl1 functions via the Co-repressor Ssn6-Tup1 and the cAMP-dependent protein kinase Tpk2. J. Mol. Biol. 309,1007-1015.

Cook, J.G., Bardwell, L., Kron, S.J., Thorner, J. (1996) Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast *Saccharomyces cerevisiae* Genes Dev 10, 2831-2848.

Cook, J.G., Bardwell, L., Thorner, J. (1997) Inhibitory and activating functions for MAPK Kss1 in the *S. cerevisiae* filamentous-growth signalling pathway. Nature 390, 85-88.

Cormack, B.P., Ghori, N., Falkow, S. (1999) An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. Science 285, 578-582.

Cullen, P.J., Sabbagh, W.Jr., Graham, E., Irick, M.M., van Olden, E.K., Neal, C., Delrow, J., Bardwell, L., Sprague, J.F.Jr. (2004) A signalling mucin at the head of the Cdc42- and MAPK-dependent filamentous growth pathway in yeast. Genes Dev 18, 1695-1708.

Cullen, P.J., Sprague, G.F.Jr. (2000) Glucose depletion causes haploid invasive growth in yeast. Proc Natl Acad Sci USA 97 (25), 13619-13624.

Cutler, N.S., Pan, X., Heitman, J., Cardenas, M.E. (2001) The TOR signal transduction cascade controls cellular differentiation in response to nutrients. Mol Biol Cell 12, 4103-4113.

De la Penas, A., Pan, S.J., Castano, I., Alder, J., Cregg, R., Cormack, B.P. (2003) Virulence-related surface glycoproteins in the yeast pathogen *Candida glabrata* are encoded in subtelomeric clusters and subject to *RAP1*- and *SIR*-dependent transcriptional silencing. Genes Dev 17, 2245-2258.

Di Como, C.J., Arndt, K.T. (1996) Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev 10, 1904-1916.

Dietrich, F.S., Voegeli, S., Brachat, S., Lerch, A., Gates, K., Steiner, S., Mohr, C., Pöhlmann, R., Luedi, P., Choi, S., Wing, R.A., Flavier, A., Gaffney, T.D, Philippsen, P. (2004) The *Ashbya gossypii* genome as a tool for mapping the ancient *Saccharomyces cerevisiae* genome. Science 304, 304-307.

Dolan, J.W., Kirkman, C., Fields, S. (1989) The yeast Ste12 protein binds to the DNA sequence mediating pheromone induction. Proc Natl Acad Sci USA 86, 5703-5707.

Dower, W.J., Miller, J.F., Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucl Acids Res 16 (13), 6127-6145.

Dünkler, A., Jorde, S., Wendland, J. (2008) An *Ashbya gossypii* cts2 mutant deficient in a sporulation-specific chitinase can be complemented by *Candida albicans CHT4* Microbiological Research 163, 701-710.

Emes, R.D., Ponting, C.P. (2001) A new sequence motif linking lissencephaly, Treacher Collins and oral-facial-digital type 1 syndromes, microtubule dynamics and cell migration. Hum Mol Genet 10, 2813-2820.

Erdman, S.E., Lin, L., Malczynski, M., Snyder, M. (1998) Pheromone-regulated genes required for yeast mating differentiation. J Cell Biol 140, 461-483.

Erdman, S.E., Snyder, M. (2001) A filamentous growth response mediated by the yeast mating pathway. Genetics 159, 919-928.

Errede, B., Ammerer, G. (1989) *STE12*, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. Genes Dev. 3, 1349-1361.

Fields, S., Herskowitz, I. (1985) The yeast *STE12* product is required for expression of two sets of cell-type-specific genes. Cell 42, 923-930.

Fujita, A., Kikuchi, Y., Kuhara, S., Misumi, Y., Matsumoto, S., Kobayashi, H. (1989) Domains of the *SFL1* protein of yeasts are homologous to Myc oncoproteins of yeast heat-shock transcription factor. Gene 85, 321-328.

Fujita, T., Tonouchi, A., Hiroko, T., Inose, F., Nagashima, T., Satoh, R., Tanaka, S. (1999) Hsl7p, a negative regulator of Ste20p protein kinase in the *Saccharomyces cerevisiae* filamentous growth-signaling pathway. Proc Natl Acad Sci USA 96, 8522-8527.

Furukawa, K., Sidoux-Walter, F., Hohmann, S. (2009) Expression of the yeast aquaporin Aqy2 affects cell surface properties under the control of osmoregulatory and morphogenetic signalling pathways. Mol Microbiol 74 (5), 1272-1286.

Gagiano, M., Bauer, F.F., Pretorius, I.S. (2002) The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*. FEMS Yeast Research 2, 433-470.

Gagiano, M., Bester, M., van Dyk, D., Franken, J., Bauer, F.F., Pretorius, I.S. (2003) Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient availability. Mol Microbiol 47 (1), 119-134.

Gagiano, M., van Dyk, D., Bauer, F.F., Lambrechts, M.G., Pretorius, I.S. (1999) Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in *Saccharomyces cerevisiae*. Mol Microbiol 31, 103-116.

Galeote, V.A., Alexandre, H., Bach B., Delobel, P., Dequin S., Blondin, B. (2007) Sfl1p acts as an activator of the *HSP30* gene in *Saccharomyces cerevisiae*. Curr Genet 52, 55-63.

Gancedo, J.M. (2001) Control of pseudohyphae formation in *Saccharomyces cerevisiae* FEMS Microbiology Reviews 25, 107-123.

Gattiker, A., Rischatsch, R., Demougin, P., Voegeli, S., Dietrich, F.S., Philippsen, P., Primig, M. (2007) *Ashbya* genome database 3.0: a cross-species genome and transcriptome browser for yeast biologists. BMC Genomics 8:9.

Gerlitz, G., Darhin, E., Giorgio, G., Franko B., Reiner, O. (2005) Novel functional features of the LisH domain, role in protein dimerization, half life and cellular localization. Cell Cycle 4 (11), 1632-1640.

Gietz, R.D., Schiestl, R.H., Willems, A.R., Woods, R.A. (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11, 355-360.

Gietz, R.D., Triggs-Raine, B., Robbins, A., Graham, K.C., Woods, R.A. (1997) Identification of proteins that interact with a protein of interest: applications of the yeast two-hybrid system. Mol Cell Biochem172, 67-79.

Gilbert, W.V., Zhou, K., Butler, T.K., Doudna, J.A. (2007) Cap-independent translation is required for starvation-induced differentiation in yeast. Science 317, 1224-1227.

Gimeno, C.J., Fink, G.R. (1994) Induction of pseudohyphal growth by overexpression of *PHD1*, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. Mol Cell Biol 14, 2100-2112.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., Fink, G.R. (1992) Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. Cell 68, 1077-1090.

Gottschling, D.E., Aparicio, O.M., Billington, B.L., Zakian, V.A. (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of PolII transcription. Cell 63, 751-762.

Govender, P., Domingo, J.L., Bester, M.C., Pretorius, I.S., Bauer, F.F. (2008) Controlled expression of the dominant flocculation genes *FLO1, FLO5* and *FLO11* in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 74, 3967-3974.

Guilliermond, A., Fontaine, M., Raffy, A. (1935) Sur l'existence dans l'Eremothecium Ashbii d'un pigment jaune se rapportant au group des flavines. Compt. Rend. Acad. Sci. Paris 201, 1077-1080.

Guo, B., Styles, C.A., Feng, Q., Fink, G. (2000) A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion and mating. Proc. Natl. Acad. Sci. USA 97, 12158-12163.

Halme, A., Bumgarner, S., Styles, C.A., Fink, G.R. (2004) Genetic and epigenetic regulation of the *FLO* gene family generates cell-surface variation in yeast. Cell 116, 405-415.

Hinnebusch, A.G. (2005) Translational regulation of *GCN4* and the general amino acid control of yeast. Ann Rev Microbiol 59, 407-450.

Hoyer, L.L, Scherer, S., Schatzman, A.R., Livi, G.P. (1995) *Candida albicans ALS1*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol Microbiol 15 (1), 39-54.

Hoyer, L.L., Payne, T.L., Hecht, J.E. (1998) Identification of *Candida albicans ALS2* and *ALS4* and localization of Als proteins to the fungal cell surface. J. Bacteriol. 180, 5334-5343.

Huang, G., Dougherty, S.D., Erdman, S.E. (2009) Conserved WCPL and CX_4C domains mediate several mating adhesion interactions in *Saccharomyces cerevisiae* Genetics 182, 173-189.

Hungerbuehler, A.K., Philippsen, P., Gladfelter, A.S. (2007) Limited functional redundancy and oscillation of cyclins in multinucleated *Ashbya gossypii* fungal cells. Eukaryotic Cell 6 (3), 473-486.

Ish-Horowicz, D., Burke, J.F. (1981) Rapid and efficient cosmid cloning. Nucl Acids Res 9 (13), 2989-2998.

Jiang, H., Zhang, Y., Sun, J., Wang, W., Gu, Z. (2008) Differential selection on gene translation efficiency between the filamentous fungus *Ashbya gossypii* and yeasts. BMC Evol Biol 8:343.

Jue, C.K. Lipke, P.N. (2002) Role od Fig2p in agglutination in *Saccharomyces cerevisiae* Eukaryotic Cell 1 (5), 843-845.

Kim, T.S., Kim, H.Y., Yoon, J.H. Kang, H.S. (2004) Recruitment of the Swi/Snf complex by Ste12-Tec1 promotes Flo8-Mss11-mediated activation of *STA1* expression. Mol Cell Biol 24 (21), 9542-9556.

Kobayashi, O., Hayashi, N., Kuroki, R., Tone, H. (1998) Region of Flo1 proteins responsible for sugar recognition. J. Bacteriol. 180, 6503-6510.

Kobayashi, O., Suda, H., Ohtani, T., Sone, H. (1996) Molecular cloning of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae* Mol. Gen. Genet. 251, 707-715.

Kobayashi, O., Yoshimoto, H., Sone, H. (1999) Analysis of the genes activated by the *FLO8* gene in *Saccharomyces cerevisiae* Curr Genet 36, 256-261.

Köhler, T., Wesche, S., Taheri, N., Braus, G.H., Mösch, H.U. (2002) Dual role of the *Saccharomyces cerevisiae* TEA/ATTS family transcription factor Tec1p in regulation of gene expression and cellular development. Eukaryotic Cell 1 (5), 673-686.

Kronstad, J.W., Holly J.A., McKay, V.L. (1987) A yeast operator overlaps an upstream activation site. Cell 50, 369-377.

Kuchin, S., Vyas, V.K., Carlson, M. (2002) Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate *FLO11*, haploid invasive growth and diploid pseudohyphal differentiation. Mol Cell Biol 22 (12), 3994-4000.

Laloux, I., Dubois, W., Dewerchin, M., Jacobs, E. (1990) *TEC1*, a gene involved in the activation of Ty1 and Ty1-mediated gene expression in *Saccharomyces cerevisiae* cloning and molecular analysis. Mol Cell Biol 10, 3541-3550.

Lamb, T.M., Mitchell, A.P. (2003) The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes *NRG1* and *SMP1* in *Saccharomyces cerevisiae* Mol Cell Biol 23, 677-686.

Liu, H., Köhler, J.R., Fink, G.R. (1994) Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science 266, 1723-1726.

Liu, H., Styles, C.A., Fink, G.R. (1993) Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science 262, 1741-1744.

Lo, H.J., Köhler, J.R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., Fink, G.R. (1997) Nonfilamentous *C. albicans* mutants are avirulent. Cell 90, 939-949.

Lo, W.S., Dranginis, A.M. (1996) *FLO11*, a yeast gene related to the *STA* genes, encodes a novel cell surface flocculin. J Bacteriol 178 (24), 7144-7151.

Lo, W.S., Dranginis, A.M. (1998) The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. Mol Biol Cell 9, 161-171.

Loeb, J.D.J., Kerentseva, T.A., Pan, T., Sepulveda-Becerra, M., Liu, H. (1999) *Saccharomyces cerevisiae* G1 cyclins are differentially involved in invasive and pseudohyphal growth independent of the filamentation mitogen-activated protein kinase pathway. Genetics 153, 1535-1546 b.

Loeb, J.D.J., Sepulveda-Becerra, M., Hazan, I., Liu, H. (1999) A G1 cyclin is necessary for maintenance of filamentous growth in *Candida albicans*. Mol Cell Biol 19, 4019-4027 a.

Lorenz, M.C., Cutler, N.S., Heitman, J. (2000) Characterization of alcohol-induced filamentous growth in *Saccharomyces cerevisiae*. Mol Biol Cell 11, 183-199 a.

Lorenz, M.C., Heitman, J. (1997) Yeast pseudohyphal growth is regulated by Gpa2, a G-protein á homolog. Embo J. 16, 7008-7018.

Lorenz, M.C., Pan, X., Harashima, T., Cardenas, M.E., Xue, Y., Hirsch, J.P., Heitman, J. (2000) The G protein-coupled receptor Gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Genetics 154, 609-622 b.

Madhani, H.D., Fink, G.R. (1997) Combinatorial control required for the specificity of yeast MAPK signalling. Science 275, 1314-1317.

Mao, X., Nie, X., Cao, F., Chen, J. (2009) Functional analysis of ScSwi1 and CaSwi1 in invasive and pseudohyphal growth of *Saccharomyces cerevisiae*. Acta Biochim Biophys Sin 41, 594-602.

McMillan, J.N., Gongtine, M.S., Sia, R.A., Theesfeld, C.L., Bardes, E.S., Pringle, J.R., Lew, D.J. (1999) The morphogenesis checkpoint in *Saccharomyces cerevisiae* cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. Mol Cell Biol 19, 6929-6939.

Miki, B.L.A., Poon, N.H., James, A.P., Seligy, V.L. (1982) Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. Journal of Bacteriology 150, 878-889.

Noble, S.M., Johnson, A.D. (2005) Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. Eukaryotic Cell 4 (2), 298-309.

Nordström, K. (1969) The spores of *Eremothecium ashbii*. J Gen Microbiol 55, 1-7.

Pan, X., Heitman, J. (1999) Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Mol Cell Biol 19, 4874-4887.

Pan, X., Heitman, J. (2000) Sok2 regulates yeast pseudohyphal differentiation via a transcription factor cascade that regulates cell-cell adhesion. Mol Cell Biol 20, 8364-8372.

Park, Y.U., Hur, H., Ka, M., Kim, J. (2006) Identification of translational regulation target genes during filamentous growth in *Saccharomyces cerevisiae*: regulatory role of Caf20 and Dhh1. Eukaryotic Cell 5 (12), 2102-2127.

Prillinger, H., Schweigkofler, W., Breitenbach, M., Briza, P., Staudacher, E., Lopandic, K., Molnár, O., Weigang, F., Ibl, M., Ellinger, A. (1997) Phytopathogenic filamentous (*Ashbya, Eremothecium*) and dimorphic fungi (*Holleya, Nematospora*) with needle-shaped ascospores as new members within the *Saccharomycetaceae*. Yeast 13, 945-960.

Roberts, R.L., Fink, G.R. (1994) Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev. 8, 2974-2985.

Rolland, F., De Winde, J.H., Lemaire, K., Boles, E., Thevelein, J.M., Winderickx, J. (2000) Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol Microbiol 38, 348-358.

Rose, M., Casadaban, M.J., Botstein, D. (1981) Yeast genes fused to â-galactosidase in *Escherichia coli* can be expressed normally in yeast. Proc Natl Acad Sci USA, 78 (4), 2460-2464.

Rupp, S., Summers, E., Lo, H.J., Madhani, H., Fink, G. (1999) MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene. The EMBO Journal 18 (5), 1257-1269.

Sambrook, J., Russell, D.W. (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schweizer, A., Rupp, S., Taylor, B.N., Röllinghoff, M., Schröppel, K. (2000) The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. Mol Microbiol 38 (3), 435-445.

Sengupta, N., Vinod, P.K., Venkatesh, K.V. (2007) Crosstalk between cAMP-PKA and MAP kinase pathways is a key regulatory design necessary to regulate *FLO11* expression. Biophys Chem 125, 59-71.

Shock, T.R., Thompson, J., Yates III, J.R., Madhani, H.D. (2009) Hog1 mitogen-activated protein kinase (MAPK) interrupts signal transduction between the Kss1 MAPK and the Tec1 transcription factor to maintain pathway specificity. Eukarytotic Cell 8 (4), 606-616.

Stahmann, K.P., Arst, H.N.Jr., Althöfer, H., Revuelta, J.L., Monschau, N., Schlüpen, C., Gätgens, C., Wiesenburg, A., Schlösser, T. (2001) Riboflavin, overproduced during sporulation of *Ashbya gossypii*, protects its hyaline spores against ultraviolet light. Env Microbiol 3 (9), 545-550.

Stahmann, K.P., Revuelta, J.L., Seulberger, H. (2000) Three biotechnological processes using *Ashbya gossypii*, *Candida famata* or *Bacillus subtilis* compete with chemical riboflavin production. Appl. Microbiol. Biotechnol. 53, 509-516.

Steiner, S., Wendland, J., Wright, M. C., Philippsen, P. (1995) Homologous recombination as the main mechanism for DNA integration and cause of rearrangements in the filamentous Ascomycete *Ashbya gossypii*. Genetics 140, 973-987.

Stratford, M., Assinder, S. (1991) Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure. Yeast 7, 559-574.

Su, C., Li, Y., Lu, Y., Chen, J. (2009) Mss11, a transcriptional activator, is required for hyphal development in *Candida albicans*. Eukaryotic Cell 8 (11), 1780-1791.

Treitel, M.A., Carlson, M. (1995) Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc Natl Acad Sci USA 92, 3132-3136.

Vallim, M.A., Miller, K.Y., Miller, B.L. (2000) *Aspergillus* SteA (sterile 12-like) is a homeodomain-C_2/H_2-Zn^{+2} finger transcription factor required for sexual reproduction. Mol Microbiol 36, 290-301.

Van der Vaart, J.M., Caro, L.H.P., Chapman, J.W., Klis, F.M., Verrips, C.T. (1995) Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. J Bacteriol 177 (11), 3104-3110.

Van Dyk, D., Pretorius, I.S., Bauer, F.F. (2005) Mss11p is a central element of the regulatory network that controls *FLO11* expression and invasive growth in *Saccharomyces cerevisiae*. Genetics 169, 91-106.

Verstrepen, K.J., Jansen, A., Lewitter, F., Fink, G.R. (2005) Intragenic tandem repeats generate functional variability. Nat. Genet. 37, 986-990.

Verstrepen, K.J., Reynolds, T.B., Fink, G.R. (2004) Origins of variation in the fungal cell surface. Nat. Rev. Microbiol. 2, 533-540.

Vinod, P.K.U., Venkatesh, K.V. (2008) A steady state model for the transcriptional regulation of filamentous growth in *Saccharomyces cerevisiae*. In Silico Biology 8, 0018.

Vyas, V.K., Kuchin, S., Berkey, C.D., Carlson, M. (2002) Snf1 kinases with different â-subunit isoforms play distinct roles in regulating haploid invasive growth. Mol. Cell. Biol. 23 (4), 1341-1348.

Wach, A., Brachat, A., Pöhlmann, R., Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10, 1793-1808.

Wang, Y., Dohlman, H.G. (2006) Pheromone-regulated sumoylation of transcription factors that mediate the invasive to mating developmental switch in yeast. Journal of Biol Chem 281 (4), 1964-1969.

Wendland, J., Ayad-Durieux, Y., Knechtle, P., Rebischung, C., Philippsen, P. (2000) PCR-based gene targeting in the filamentous fungus *Ashbya gossypii*. Gene 242, 381-391.

Wendland, J., Walther, A. (2005) *Ashbya gossypii*: a model for fungal developmental biology. Nat Rev Microbiol 3, 421-429.

Wilson, W.A., Hawley, S.A., Hardie, D.G. (1996) Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under

derepressing conditions, and this correlates with a high AMP:ATP ratio. Curr Biol 6, 1426-1434.

Wright, M. C., Philippsen, P. (1991) Replicative transformation of the filamentous fungus *Ashbya gossypii* with plamids containing *Saccharomyces cerevisiae* ARS elements. Gene 109, 99-105.

Wu, C., Leeuw, T., Leberer, E., Thomas, D.Y., Whiteway, M. (1998) Cell cycle- and Cln2p-Cdc28p-dependent phosphorylation of the yeast Ste20p protein kinase. J Biol Chem 273, 28107-28115.

Xiao, J.H., Davidson, I., Matthes, H., Garnier, J.M. Chambon, P. (1991) Cloning, expression, and transcriptional properties of the human enhancer factor *TEF-1*. Cell 65, 551-568.

Xue, Y., Batlle, M., Hirsch, J.P. (1998) *GPR1* encodes a putative G protein-coupled receptor that associates with the Gpa2p Gá-subunit and functions in a Ras-independent pathway. EMBO J. 17, 1996-2007.

Yoon, S., Xiu, H., Swanson, M.J., Hinnebusch, A.G. (2003) Recruitment of SWI/SNF by Gcn4p does not require Snf2p or Gcn5p but depends strongly on SWI/SNF integrity, SRB mediator, and SAGA. Mol Cell Biol 23, 8829-8845.

Yuan, Y.L., Fields, S. (1991) Properties of the DNA-binding domain of the *Saccharomyces cerevisiae* Ste12 protein. Mol Cell Biol 11, 5910-5918.

Yuan, Y.O., Stroke, I.L., Fields, S. (1993) Coupling of cell identity to signal response in yeast: interaction between the alpha1 and STE12 proteins. Genes Dev 7, 1584-1597.

Zhang, M., Bennett, D., Erdman, S.E. (2002) Maintenance of mating cell integrity requires the adhesin Fig2p. Eukaryotic Cell 1 (5), 811-822.

Zupancic, M. L., Frieman, M., Smith, D., Alvarez, R. A., Cummings, R. D., Cormack, B. P. (2008) Glycan microarray analysis of *Candida glabrata* adhesion ligand specificity. Mol Microbiol 68: 547-559.

Choice of topic and experiments of this work has been done in communication with Prof. Dr. Wendland.

7. List of Abbreviations

2YT	2 x yeast extract tryptone
aa	amino acids
AFM	*Ashbya* Full Medium
ALS	agglutinine-like-sequences
ARS	autonomous replicating sequence
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CSM	complete supplement mixture
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide-tri-phosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPA	epithelial adhesion
FRE	filamentation/invasion response element
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GTP	guanosine-tri-phosphate
HOG	high osmolarity glycerol
Hsf	heat shock factor
IRES	internal ribosome entry site
LisH	Lissencephaly type-1-like homology motif
MAPK	mitogen-activated protein kinase
MU	Miller Units
Nc RNA	non coding RNA
NLS	nuclear localization sequence
OD	optical density
ONPG	*ortho*-Nitrophenyl-â-galactoside
ORF	open reading frame
PCR	polymerase chain reaction
PEG	poly ethylene glycol

pFA	plasmid for functional analysis
PKA	protein kinase A
PMSF	phenylmethylsulfonyl fluoride
PRE	pheromone response element
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	rounds per minute
RT-PCR	reverse transcriptase PCR
SAP	shrimp alkaline phosphatase
SCF	Skp1, Cdc53 (Cullin), F-box proteins
SDS	sodium dodecyl sulfate
Std/Stdev	standard deviation
STE	sorbitol-TRIS-EDTA
STM	saccharose-TRIS-$MgCl_2$
TAE	TRIS-Acetic acid-EDTA
TCS	Tec1-binding site
TE	Tris-EDTA
TEA/ATTS	TEF-1, Tec1, AbaA/ AbaA, Tef-1, Tec1, Scalloped
TOR	target of rampamycin
TRIS	tris(hydroxymethyl)aminomethane
UAS	upstream activating sequence
UTR	untranslated region
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
YNB	yeast nitrogen base
YPD	yeast extract peptone dextrose

8. Scientific publications

Scientific papers

Grünler, A., Walther, A., Lämmel, J., Wendland, J. Analysis of flocculins in *Ashbya gossypii* reveals *FIG2* regulation by *TEC1*. Fungal Genet Biol **47** (7), 619-628.

Rispail, N., Soanes, D.M., Ant, C., Czajkowski, R., **Grünler, A**., Huguet, R., Perez-Nadales, E., Poli, A., Sartorel, E., Valiante, V., Yang, M., Beffa, R., Brakhage, A.A., Gow, N.A.R., Kahmann, R., Lebrun, M., Lenasi, H., Perez-Martin, J., Talbot, N.J., Wendland, J., Di Pietro, A. (2009) Comparative genomics of MAP kinase and calcium-calcineurin signalling components in plant and human pathogenic fungi. Fungal Genet Biol **46** (4), 287-298.

Wälti, M.A., Walser, P.J., Thore, S., **Grünler, A.**, Ban, N., Künzler, M., Aebi, M. (2008) Structural basis for chitotriose coordination of CGL3, a novel galectin-related protein from *Coprinopsis cinerea*. J Mol Biol **379** (1), 146-159.

Wälti, M.A., Villalba, C., Buser, R.M., **Grünler, A.**, Aebi, M., Künzler, M. (2006) Targeted gene silencing in the model mushroom *Coprinopsis cinerea* (*Coprinus cinereus*) by expression of homologous hairpin RNAs. Eukaryotic Cell **5**, 732-744.

Wöstemeyer, J., **Grünler, A.**, Schimek, C., Voigt, K. (2005) Genetic regulation of carotenoid biosynthesis in fungi. Appl Mycol Biotechnol **5**, 257-274.

Scientific talks

Signalpath midterm meeting in Ljubliana, May 2008, scientific workshop and scientific talk

Signalpath meeting in Copenhagen, October 2008, scientific workshop and scientific talk

Signalpath meeting in Aberdeen, May 2009, scientific workshop and scientific talk

Signalpath final meeting in Berlin, October 2009, final report

Carlsberg Laboratory Seminar, November 2009

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